The Effects of Nitric Oxide and Nitric Oxide-Related Species on the Viability of Cell Types Critical to Atherosclerosis: Obligatory Role of Peroxynitrite in Cell Death

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Declaration

I hereby declare that the data published in this thesis are the result of my own work, carried out under the supervision of Drs Ian L. Megson and Adriano. G Rossi at The University of Edinburgh, and that this thesis has been completed entirely by myself and has not previously been submitted for any other degree or qualification.

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Abstract

The endogenous free radical signalling molecule, nitric oxide (NO), is the mediator of many physiological processes including control of vasomotor tone, and the inhibition of smooth muscle cell (SMC) proliferation and platelet activation. Additionally, NO plays an important role in the regulation of the inflammatory response through its ability to influence apoptosis in a variety of cells. This fundamental process governing cell survival is crucial to ensuring the successful resolution of the inflammatory response as apoptotic inflammatory cells are removed from a site of tissue injury by non-inflammatory phagocytosis. NO has both pro- and anti-apoptotic properties depending on its concentration, the NO-related species generated and the cell type in question. Pharmacological manipulation of apoptosis by NO may aid the resolution of inflammation during chronic inflammatory disorders such as atherosclerosis.

NO is often reported as having paradoxical effects in many biological settings. This may be due to the formation of NO-related species *in vivo*: NO combines rapidly with superoxide anion to generate peroxynitrite ($ONOO^-$) and with endogenous thiols to form S-nitrosothiols (RS-N=O). Both $ONOO^-$ and RS-N=O have biological properties that are independent of the liberation of NO radical *per se*.

Characterisation of the NO-related species generated by the NO donor compounds 1,2,3,4,-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-chloride (GEA-3162), diethylamine diazeniumdiolate (DEA/NO), (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO), S-nitroso-N-valeryl-D-penicillamine (SNVP) and S-nitrosoglutathione (GSNO), was carried out by a combination of electrochemistry and electron paramagnetic resonance. Results revealed the diazeniumdiolates, DEA/NO and DETA/NO, spontaneously liberate NO

radical in solution. However, GEA-3162 was found to release NO and O_2^- concomitantly, and therefore, should be regarded as a $ONOO^-$ generator. SNVP and GSNO release only small amounts of free NO in solution, however, the mechanism of action of these compounds is likely to also involve the transfer of NO^+.

The effect of each of these compounds on cell viability was investigated in human monocyte-derived macrophages (Mφ) and an aortic SMC line. In both Mφ and SMC, only the $ONOO^-$ generator, GEA-3162, induced cell death. Analysis by flow cytometry revealed GEA-3162 caused cell surface phosphatidylserine exposure characteristic of apoptosis. Only a prolonged delivery of NO radical was able to genuinely inhibit SMC proliferation. These results demonstrate, that contrary to previous reports, NO *per se* is incapable of inducing cell death.

Finally, apoptotic cell death induced by GEA-3162 in human monocyte-derived Mφ was inhibited by pre-treatment of the cells with a low dose of the NO-releasing compound, DETA/NO. This effect was enhanced by augmenting cGMP levels using a direct guanylate cyclase stimulator, BAY 41-2272. Pre-treatment with DETA/NO failed to protect SMC against GEA-3162-induced cell death, irrespective of inclusion of BAY 41-2272. These results demonstrate that pre-conditioning of Mφ with cGMP protects them against subsequent cell death.

Taken together, these studies demonstrate that the outcome of any NO-induced response depends on the concentration and precise NO-related species generated in the microenvironment, as well as the nature of the target cell. Exploiting these differences as a therapeutic strategy for the treatment of atherosclerosis may be possible with the advent of cell-specific NO donor compounds.

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working,' moanings, but as they always greeted this news with cheer and assured me we'd get there in the end!

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Abbreviation	Definition
1400W	N-(3-(aminomethyl)benzyl) acetamidine dihydrocholride
ANOVA	Analysis of variance
Apaf	Apoptotic protease activating factor 1
ApoE	Apolipoprotein E
Arg	Arginine
ATP	Adenosine triphosphate
AUC	Area under the curve
BAoSMC	Bovine aortic smooth muscle cells
BCA	Bicinchoninic acid
BH_4	Tetrahydrobiopterin
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}/CAM	Calcium calmodulin
$CaCl_2$	Calcium chloride
CAD	caspase-activated deoxyribonuclease
CAMP	Cyclic adenosine monophosphate
cGMP	3',5'-Cyclic guanosine monophosphate
CH_2	Methylene gruop
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
Cu/ZnSOD	Copper/zinc SOD
Cu^+	Copper (I) ions
Cu^{2+}	Copper (II) ions
Cys	Cysteine
DAN	2,3-diaminonaphthalene
DD	Death domain
DEA/NO	2-(N,N-diethyamino)-diazeniumdiolate
DED	Death effector domain
DETA/NO	(Z)-1-[2-(2-aminoethy)-N-(2-ammonioethy)amino]diazen-1-ium-1,2-diolate
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDRF	Endothelium-derived relaxing factor
EDTA	Tetraacetic acid
ELISA	Enzyme-linked immunosorbant
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FADD	Fas-associated protein with death domain
Fas-L	Fas ligand
FCS	Foetal calf serum
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
FITC	Fluorescein isothiocyanate
FLIP	FLICE-like inhibitory protein
FMN	Flavin mononucleotide
FS	Forward scatter
GSNO	S-nitrosoglutathione
GTN	Glyceryl trinitrate
γ-GT	γ-glutamyltranspeptdase
GTP	Guanosine 5'-triphosphate

H_2O	Water
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
Hsp	Heat shock proteins
IAP	Inhibitor of apoptosis protein
IBMX	3-Isobutyl-1-methylxanthine
iCAD	Inhibitor of caspase-activated deoxyribonuclease
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inflammatory nitric oxide synthase
IP_3	Inositol triphosphate
KPSS	High potassium physiological saline solution
$L^{\bullet}$	Alkyl radical
L-Arg	L-arginine
LDH	Lactate dehydrogenase
LDL	Low density liopoprotein
L-NMMA	N^G-monomethyl-L-arginine
$LO^{\bullet}$	Alkoxyl radical
$LOO^{\bullet}$	Peroxyl radical
LPS	Lipopolysaccharide
LY 83583	6-anilino-5,8-quinolinequinone
MAPK	Mitogen activated kinase
MCP-1	Monocyte chemoattractive protein-1
M-CSF	Macrophage colony stimulating factor
Mg^{2+}	Magnesium ions
MMP	Matrix matalloproteinase
MPO	Myloperoxidase
MRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium hydrocarbonate
NANC	Non-adrenergic, non-cholinergic
NaOH	Sodium hydroxide
NAT	2,3-naphthotriazole
NFκB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric Oxide
NO^+	Nitrosium ion
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NOx	NO-related species
NSB	Non-specific binding
O_2	Oxygen
O_2^-	Superoxide anion
ODQ	1-*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	peroxynitrite
ONOOH	Peroxynitrous acid
ox-LDL	Oxidation-modified low density lipoprotein
PAPA/NO	(Z)-1-[N-(3-Ammoniopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate

PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PE	Phenylephrine
pHA	p-hydroxyphenylacetaldehyde
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent kinase
PLC	Phospholipase C
PNPP	*p*-nitrophenol phosphate
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSS	Physiological saline solution
ROS	Reactive oxygen species
RPMI	Roswell Park memorial Institute
RS-N=O	S-nitrosothiol
RT	Room temperature
SEM	Standard error of the mean
Ser	Serine
sGC	Soluble guanylate cyclase
SIN-1	3-morpholinosyndnonimine
SMC	Smooth muscle cell
SNAP	S-nitroso-N-acetlypenicillamine
SNP	Sodium nitroprusside
SNVP	S-nitroso-N-valerly-penicillamine
SOD	Superoxide dismutase
SPER/NO	Spermine diazeniumdiolate
SS	Side scatter
Tempone-H	1-Hydroxy-2,2,6,6-tetramethy-4-oxo-piperidine
TGF-β	Transforming growth factor β
TMB	Tetra-methylbenzidine
TNF-R1	Tumour necrosis factor receptor 1
TNF-α	Tumour necrosis factor α
TRADD	Tumour necrosis factor receptor 1-associated protein with death domain
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TUNEL	Deoxynucleotidyl transferae (TdT)-mediated dUTP-biotin nick end-labelling
Tyr	Tyrosine
VASP	Vasodilator-stimulated phosphoprotein
VCAM-1	Vascular cell adhesion moleule-1
VSMC	Vascular smooth muscle cell
YC-1	3-(5'-hydroxymethy-2'-furyl)-1-benzyl-indazol

Chapter One

Introduction

1. INTRODUCTION

1.1 Introduction

The endogenous free radical, nitric oxide (NO), is a signalling molecule responsible for mediating various physiological and pathophysiological processes including modulation of vascular tone, inhibition of platelet and inflammatory cell activation and adhesion, and neurotransmission at non-adrenergic, non-cholinergic (NANC) neurones (Moncada et al. 1991; Quinn et al. 1995; Ignarro et al. 1999). In addition, NO plays an important role in the regulation of inflammatory processes through its ability to manipulate apoptosis in a variety of cell types (Nicotera et al. 1997; Fiscus et al. 2002; Taylor et al. 2003). However, due to the free radical status of NO, it rapidly combines with reactive oxygen species present in the milieu, and with various cellular components such as cysteine residues on proteins, to form a variety of NO-related species *in vivo*. The net outcome of any NO-regulated process therefore critically depends not only on the cell type in question, but also on the NO concentration liberated and the precise NO-related species formed in the microenvironment.

Alterations in both the synthesis and bioavailability of NO are likely to influence cellular processes occurring in a wide range of diseases, including those with a chronic inflammatory component, such as atherosclerosis. Atherosclerosis is a multi-factorial disease with a complicated aetiology. Atherosclerosis and the associated acute clinical events, such as stroke and myocardial infarction, are a major

cause of morbidity and mortality in industrialised nations and, despite decades of research, the clinical consequences of atherosclerosis still account for vast numbers of adult deaths in Western society (The American Heart Association Statisical Update 2005). Atherosclerosis has been the most common underlying cause of adult death in the United States almost every year since 1900 and mortality rates from atherosclerosis continue to exceed those for cancer, infectious diseases and trauma (Callow 2002; The American Heart Association Statisical Update 2005).

Atherosclerosis is characterised by the formation of lipid-rich plaques in the sub-endothelial space of major conduit vessel such as the coronary arteries and aorta (Ross 1993; Davies 1997). It is widely recognised that there is an inflammatory component to the pathogenesis and progression of this condition, and atherosclerosis is now considered to be the consequence of a chronic, low-grade inflammatory response (Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002). Progression of the inflammatory response depends on the balance between the recruitment and activation of inflammatory cells and of their subsequent removal from the inflammatory milieu by phagocytosis. Apoptosis, or programmed cell death, is a fundamental process governing cell survival and is critically involved in ensuring the successful resolution of the inflammatory response (Kerr et al. 1972; Haslett 1997; Wyllie 1997). Apoptotic cells are instantly recognised for non-inflammatory clearance by phagocytes (e.g. macrophages) and removed from the vicinity of inflammation without the release of their histotoxic, pro-inflammatory contents (Savill et al. 1993). Dysregulation of apoptosis or of phagocytic clearance

mechanisms may have drastic consequences for the resolution of inflammation, resulting in an exacerbation of the inflammatory response (Haslett 1997).

1.2 Nitric Oxide

It is now over quarter of a century since the importance of the vascular endothelium was realised. During the response to various agonists, including acetylcholine, bradykinin, and thrombin, the endothelium was observed to release an endothelium-derived relaxing factor (EDRF) which induced both vascular relaxation (Furchgott and Zawadzki 1980; Furchgott 1984; Furchgott et al. 1984; Furchgott et al. 1987; Amezcua et al. 1988) and a potent inhibition of platelet aggregation and adhesion (Alheid et al. 1987; Busse et al. 1987; Furlong et al. 1987; Radomski et al. 1987a; Bhardwaj et al. 1988; Hogan et al. 1988; Macdonald et al. 1988; Sneddon and Vane 1988). Subsequently, EDRF was found to have chemical and pharmacological properties indistinguishable from the inorganic free radical, nitric oxide (NO; Furchgott and Zawadzki 1980; Ignarro et al. 1987a; Ignarro et al. 1987b; Palmer et al. 1987; Ignarro et al. 1988a; Ignarro et al. 1988b). Since then, it has become increasingly clear that NO is the signalling molecule responsible for a wide range of diverse physiological and pathophysiological processes. NO is now known to control vascular smooth muscle tone (Amezcua et al. 1989; Ekelund and Mellander 1990; Faraci 1990; Gardiner et al. 1990; Kelm and Schrader 1990; Tare et al. 1990), to inhibit platelet and inflammatory cell activation (Radomski et al. 1990; May et al. 1991; Radomski et al. 1991; Kanwar and Kubes 1995; Quinn et al. 1995; Armstrong 2001), to be a bi-functional regulator of apoptosis in a variety of cell types (Albina et

al. 1993; Nicotera et al. 1997; Taylor et al. 2003) and to be a transmitter at non-adrengeric non-cholinergic (NANC) synapses (Graham and Sneddon 1993; Hernandez et al. 1995; Postorino et al. 1995; Simonsen et al. 1995; Yunker and Galligan 1996).

1.2.1 NO Synthesis

NO is synthesised endogenously by three isoforms of the enzyme NO synthase (NOS; Charles et al. 1996). A series of studies in the early 1990s isolated three NOS isoforms: endothelial NOS (eNOS or NOS III: (Pollock et al. 1991; Lamas et al. 1992; Marsden et al.; Nishida et al. 1992), neuronal NOS (nNOS or NOS I: (Bredt et al. 1991a), and inducible NOS (iNOS or NOS II: (Yui et al. 1991a; Sherman et al. 1993; Wood et al. 1993). These isoenzymes are cytochrome P450-reductase related enzymes (Bredt et al. 1991b; White and Marletta 1992) which catalyse the formation of NO and L-citrulline (L-Cit) from L-arginine (L-Arg) and oxygen (O_2; Mayer et al. 1989; Johns and Rengasamy 1991; Bush et al. 1992a; Bush et al. 1992b). eNOS and nNOS are constitutively expressed, are calcium/calmodulin (Ca^{2+}/CAM) dependent (Bredt and Snyder 1990; Schmidt et al. 1992a; Schmidt et al. 1992b) and synthesise relatively low NO concentrations (in the pM range). iNOS is Ca^{2+}/CAM independent (Yui et al. 1991b) and expressed in response to various cytokines and bacterial endotoxins to generate high NO concentrations (nM – μM; Moncada et al. 1991; Berdeaux 1993).

NOS isoenzymes exist as homodimers, with each monomer containing an oxygenase domain linked to a reductase domain (Griffith and Stuehr 1995; Alderton

et al. 2001). The reductase domain contains binding sites for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NADPH; Marletta 1994; Ghosh and Stuehr 1995), whilst the oxygenase domain contains the binding sites for L-Arg, and the co-factor tetrahydrobiopterin (BH_4), plus a haem prosthetic group (Crane et al. 1998; Raman et al. 1998). NADPH donates electrons which flow from the reductase domain via the flavin cofactors to the haem group of the oxygenase domain, where they reduce ferric (Fe^{3+}) haem to the ferrous (Fe^{2+}) form, allowing O_2 to bind (Siddhanta et al. 1996; Adak et al. 1999; Poulos et al. 1999). NO is then formed by the oxidation of L-Arg via the formation of the intermediate, N^{ω}-hydroxy-L-arginine (Korth et al. 1994; Alderton et al. 2001). BH_4 is a required co-factor for NO synthesis by NOS (Tayeh and Marletta 1989; Kilbourn 1991; Mayer et al. 1991; Gross and Levi 1992; Sakai et al. 1993; Schoedon et al. 1993), and recent studies have demonstrated that a lack of BH_4 leads to uncoupling of NOS with a resulting generation of superoxide anion (O_2^-) rather than NO (Xia and Zweier 1997; Vasquez-Vivar et al. 1998; Xia et al. 1998; Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1999b; Vasquez-Vivar et al. 2002). Figure 1.1.

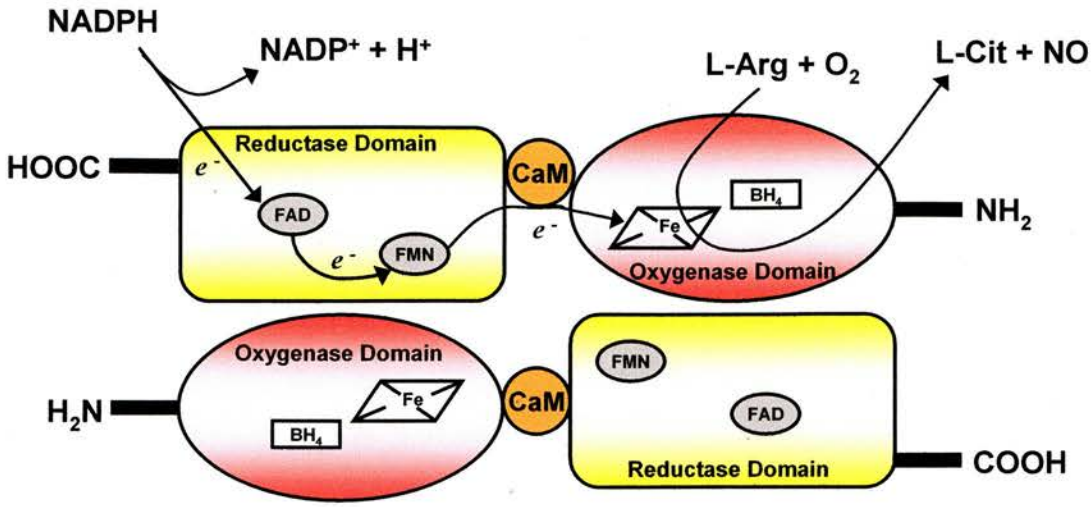

Figure 1.1 Schematic Representation of The Structure of NOS

Electrons flow from NADPH through the flavins (FAD and FMN) of the reductase domain of one subunit via calmodulin (CaM) to the haem group of the oxygenase domain in the other subunit. Tetrahydrobiopterin (BH4) is a required co-factor for the synthesis of NO from L-arginine (L-Arg). Adapted from Alderton et al. (2001).

1.2.2 Mechanism of Action of NO

The classical pathway by which NO exerts its effects is via activation of the enzyme soluble guanylate cyclase (sGC), resulting in the conversion of guanosine 5'-triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP) (Schroder et al. 1985; Feelisch and Noack 1987; Mulsch et al. 1987; Mayer and Bohme 1989; Mayer et al. 1989; Palacios et al. 1989; Ishii et al. 1991; Schmidt 1992; Moro et al. 1996).

1.2.2.1 NO-Mediated Activation of sGC

NO diffuses from the site of production, for example endothelial cells, and passes through the plasma membrane of target cells such as smooth muscle cells, platelets or inflammatory cells without the need for a membrane bound receptor or transporter. Following diffusion into target cells, NO activates sGC present in the cytosol. sGC exists as a heterodimer of α and β monomer subunits (Kamisaki et al. 1986; Harteneck et al. 1990). Each sGC monomer consists of a catalytic domain at the C-terminal, a central dimerisation region and a haem binding domain at the N-terminal (figure 1.2; Zhao and Marletta 1997; Andreopoulos and Papapetropoulos 2000). NO binds the haem moiety of sGC to form a nitrosyl-haem complex (Ignarro et al. 1986), increasing the enzymatic activity by up to 400 fold (Stone and Marletta 1996). A histidine residue (His-105) on the β subunit has been identified as critical for haem-dependent NO binding (Wedel et al. 1994; Zhao et al. 1998), however several other residues including Cys-78 & 214 (Friebe et al. 1997), Tyr-125, Arg-139, (Schmidt et al. 2004) and Ser-137 (Schmidt et al. 2005) have more recently been identified as playing important roles during NO-activation of sGC, as has the requirement for a complete heterodimer (Foerster et al. 1996). Formation of the nitrosyl-haem complex leads to activation of the enzyme as the bond with the axial histidine residues breaks, resulting in changes to the tertiary and quaternary structure of the enzyme (Deinum et al. 1996; Hobbs 1997). This conformational change in structure results in a marked increase in the V_{max} of sGC and a reduction in the K_m of the substrate GTP from ~100 μM - ~ 50 μM, hence resulting in the formation of cGMP (Wolin et al. 1982). Figure 1.2.

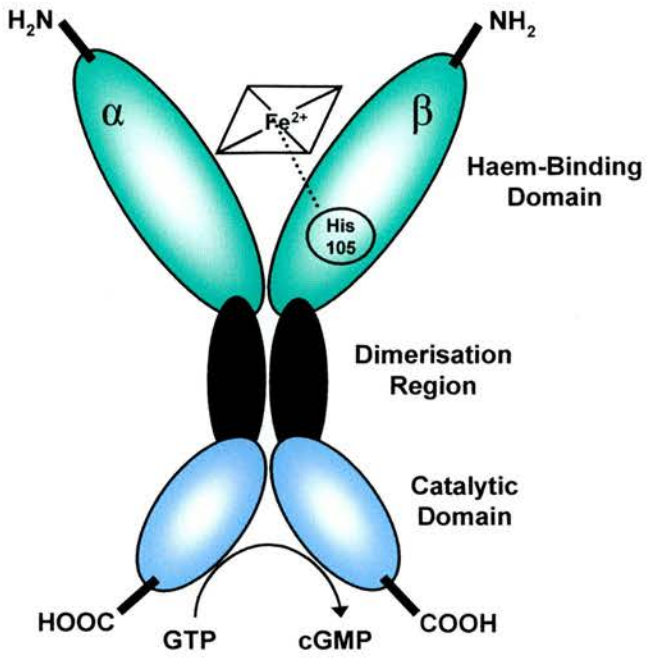

Figure 1.2 Schematic Representaion of the Structure of sGC

sGC consisits of three domains as indicated. A hisitidine residue (His 105) on the β subunit is amongst several residues thought to be critical for the binding of NO to the haem prosthetic group. Adapted from Hobbs (1997).

1.2.2.2 cGMP-Mediated Responses

Elevation of cGMP mediates the cellular effects of NO principally via activation of cGMP-dependent kinase (PKG) (Carvajal et al. 2000). PKG is a serine/theronine protein kinase, which exists in two isoforms – PKG I and PKG II (Vaandrager and de Jonge 1996). PKG acts principally to inhibit Ca^{2+} signalling, by triggering a reduction in intracellular calcium concentration, $[Ca^{2+}]_i$ in platelets (Geiger et al. 1992; Menshikov et al. 1993; Heemskerk et al. 1994) and smooth muscle cells (Rashatwar et al. 1987; Chen and Rembold 1992; McDaniel et al. 1992).

PKG has been demonstrated to phosphorylate a serine residue on the inositol triphosphate (IP_3) receptor of the sarcoplasmic reticulum, preventing Ca^{2+} efflux into the cytoplasm, thereby decreasing $[Ca^{2+}]_i$ (Komalavilas and Lincoln 1994; Cavallini et al. 1996; El-Daher et al. 2000; Murthy and Zhou 2003). cGMP can also inhibit the activity of phospholipase C (PLC), the enzyme responsible for the generation of the second messenger, IP_3 (Lincoln and Cornwell 1993; Carvajal et al. 2000). In addition to its effects on the IP_3 system, PKG has been proposed to elicit decreases in $[Ca^{2+}]_i$ via various other direct and indirect mechanisms. In smooth muscle cells, direct inhibition of membrane Ca^{2+} channel activity (Clapp and Gurney 1991; Quignard et al. 1997), activation of Ca^{2+}/ATPase pumps in the plasma membrane (Furukawa et al. 1988) and the sarcoplasmic reticulum (Cornwell et al. 1991; Trepakova et al. 1999), and indirect reduction of the sensitivity of the contractile filaments to Ca^{2+} (Carvajal et al. 2000) have been proposed as additional actions of PKG. PKG may also phosphorylate additional cellular proteins including heat shock proteins (Hsp), in both smooth muscle cells (Carvajal et al. 2000) and platelets (Butt et al. 2001). In platelets, vasodilator-stimulated phosphoprotein (VASP) is phosphorylated by PKG (Halbrugge et al. 1990; Butt et al. 1994; Meinecke et al. 1994). VASP regulates actin polymerisation and organisation in platelets (Reinhard et al. 1992; Reinhard et al. 2001). Phosphorylation of this protein decreases its ability to interact with actin filaments (Harbeck et al. 2000) and this correlates with platelet GPIIb/IIIa receptor inhibition (Horstrup et al. 1994). Overall, these combined actions of PKG result in inhibition of platelet activation and reduced

smooth muscle contraction (vasodilatation). Addionally, cGMP has cytostatic effects in smooth muscle cells (see section 1.4.2.2.1).

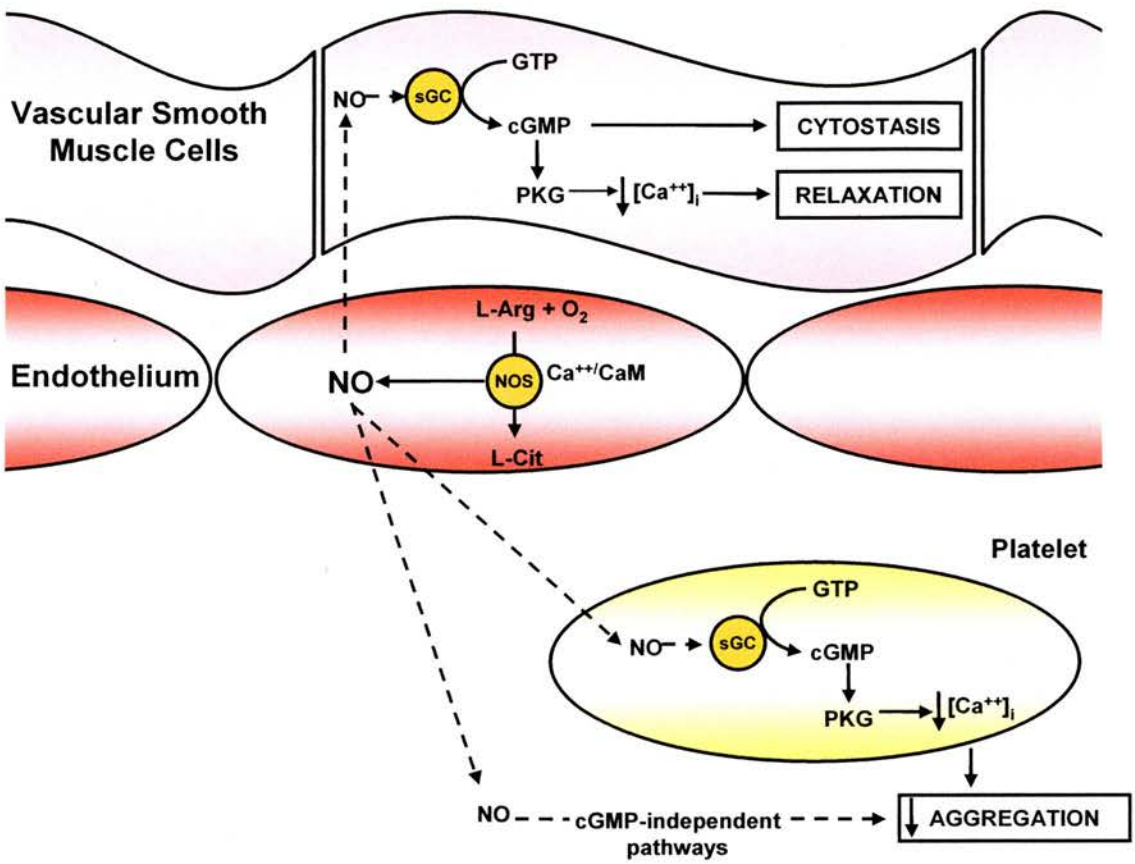

Figure 1.3 The Actions of Endothelial NO on Smooth Muscle Cells and Platelets

NO diffuses from endothelial cells to activate soluble guanylate cyclase (sGC) in target cells. sGC converts GTP to cGMP. cGMP activates cGMP-dependent protein kinase G (PKG) to cause a decrease in intracellular calcium concentration $[Ca^{2+}]_i$. Decreasing $[Ca^{2+}]_i$ results in inhibition of aggregation in platelets, and in VSMC results in relaxation and hence, vasodilatation. Extracellular NO can also activate cGMP-independent pathways available to platelets (see section 1.2.2.4). Additionally, cGMP has cytostatic affects in smooth muscle cells.

1.2.2.3 Phosphodiesterases

cGMP is rapidly inactivated by phosphodiesterase enzymes (PDEs), which convert cyclic nucleotides (both cGMP and cyclic adenosine monophosphate; cAMP) to their inactive 5'-nucleotides (Moncada et al. 1991; Maurice et al. 2003). A large family of PDE isoforms exists in mammals, (Beavo 1995; Soderling and Beavo 2000) most of which will hydrolyse both cGMP and cAMP (Matsumoto et al. 2003). Most cells contain representatives of several isoforms of the PDE family in varying amounts, however, arguably the most relevant PDE in the cardiovascular system is PDE V, which specifically hydrolyses cGMP (Thomas et al. 1990; McAllister-Lucas et al. 1995), and is expressed abundantly in vascular smooth muscle cells and platelets (Matsumoto et al. 2003). Recently it has been demonstrated that PKG and protein kinase A (PKA: the enzyme activated by cAMP) can phosphorylate PDE enzymes in both smooth muscle cells and platelets (Mehats et al. 1999; Corbin et al. 2000; Liu et al. 2000; Murthy et al. 2002; Mullershausen et al. 2003). This may provide a long term feedback mechanism to modulate the responses elicited by cyclic nucleotides.

1.2.2.4 NO-Mediated cGMP-Independent Responses

Recent studies have established that, in addition to the cGMP-dependent mechanism of action, NO can also act via a number of cGMP-independent pathways. The existence of such cGMP-independent pathways has been demonstrated in several biological systems, most notably during inhibition of platelet aggregation (Gordge et al. 1998; Tsikas et al. 1999; Sogo et al. 2000a; Crane et al. 2002; Crane et

al. 2005), and regulation of inflammatory cell apoptosis (Ward et al. 2000). The site of NO production (or delivery in the case of NO donor drugs) appears, to some degree, to determine whether NO acts via cGMP-dependent or cGMP-independent pathways. This appears to be the case in platelets, with cGMP-independent pathways only becoming activated by NO generated extracellularly (Sogo et al. 2000a; Crane et al. 2005); NO-mediated smooth muscle relaxation shows a similar cGMP-independent component, but its contribution is less significant than in platelets (Miller et al. 2004).

The existence and understanding of NO-mediated cGMP-independent pathways has been significantly advanced by the development of the potent and specific inhibitor of sGC, 1-*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; Garthwaite et al. 1995; Schrammel et al. 1996). ODQ oxidises the ferrous haem of sGC to the ferric form, vastly reducing NO-stimulated sGC activity (Zhao et al. 2000). ODQ offers significant advantages over previously used sGC inhibitors such as methylene blue and LY 83583 (6-anilino-5,8-quinolinequinone) which can generate superoxide anion (O_2^-) and interfere with prostanoid synthesis (Martin et al. 1989; Wolin et al. 1990; Hasegawa et al. 2004).

1.2.3 Chemistry of NO

The simplicity of the NO molecule belies the complex chemistry of this versatile signalling mediator. Due to its free radical nature, NO can rapidly with molecular oxygen and reactive oxygen species (ROS) to form a variety of NO-related species, which may have indirect biological effects of their own. There are

many reports in the literature of NO having opposing effects in various systems. For example, is has both antioxidant and pro-oxidant properties (Goss et al. 1995; Rubbo et al. 1995; Struck et al. 1995; Joshi et al. 1999; Wei et al. 2000), cytotoxic and cytoprotective actions (Polte et al. 1997; Stefanelli et al. 1999; Mason et al. 2000; Keira et al. 2002; Hattori et al. 2004) and can be pro- or anti-apoptotic (Ward et al. 2000; Kim et al. 2001; Fiscus et al. 2002). This dual modality, and apparent paradox of NO activity may be explained, at least in part, by the production of intermediary NO-related species, with the ultimate outcome of any NO-mediated response depending on the precise NO-related species formed in the microenvironment, as well as by the concentration of NO generated and the nature of the target cell type.

1.2.3.1 Reaction of NO with Molecular Oxygen

The biological half-life of NO *in vivo* is extremely short because it reacts readily with a host of other molecules present in the milieu. NO is oxidised by molecular oxygen (O_2) in the gaseous or aqueous phases, to form nitrogen dioxide (NO_2: equation 1; Lewis and Deen 1994; Wink et al. 1994). NO_2 will react with water (H_2O) to form nitrite (NO_2^-) and nitrate (NO_3^-: equation 2; Butler et al. 1995), but preferentially reacts with NO to form N_2O_3 (equation 3; Wink et al. 1993; Espey et al. 2001). N_2O_3 is a powerful S-nitrosating species (see section 1.2.3.2. below) and will also react with H_2O to generate NO_2^- (equation 4).

$$NO + O_2 \longrightarrow NO_2 \quad (1)$$

$$2NO_2 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \quad (2)$$

$$NO_2 + NO \longrightarrow N_2O_3 \quad (3)$$

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+ \quad (4)$$

1.2.3.2 Reaction of NO with Thiol Groups

N_2O_3 is a particularly important NO-related mediator as it is the most relevant nitrosonium ion (NO^+) donor in biological systems (Wink and Mitchell 1998). NO^+ is exceedingly reactive *in vivo*, and 'free' NO^+ is never specifically liberated at physiological pH; instead it is transferred to reduced cysteine residues in proteins, or thiol groups of low molecular weight compounds, resulting in the formation of S-nitrosothiols (RS-N=O: equation 5; Kharitonov et al. 1995; Liu et al. 1998; Jourd'heuil et al. 2003). For example, the S-nitrosation of the plasma protein albumin, leads to the formation of S-nitrosoalbumin (Stamler et al. 1992; Keaney et al. 1993; Marley et al. 2001). The production of S-nitrosothiols, including S-nitrosoalbumin, has recently been proposed as a mechanism for the formation of a slow release *in vivo* NO reservoir which may prolong NO bioavailability (Simon et al. 1993; Scharfstein et al. 1994; Minamiyama et al. 1996; Crane et al. 2005; Orie et al. 2005).

$$N_2O_3 + RSH \longrightarrow RS\text{-}N{=}O + H^+ + NO_2^- \quad (5)$$

S-Nitrosothiols have many of the actions of NO, including the induction of vasodilatation (Keaney et al. 1993; Kukreja et al. 1993; Rayner et al. 2004) and apoptosis (Xie et al. 2003; Yasinska et al. 2004), and the inhibition of platelet aggregation (Simon et al. 1993; Langford et al. 1994; Gordge et al. 1998; Megson et al. 2000; Miller et al. 2003). The precise mechanism for these actions is not yet fully understood and may occur without the liberation of NO *per se* (Kowaluk and Fung

1990; Ceron et al. 2001), but via rapid transnitrosation reactions resulting in the transfer of NO^+ from one thiol moiety to another (equation 6; Scharfstein et al. 1994; Sexton et al. 1994; Askew et al. 1995; Dicks and Williams 1996; Gorren et al. 1996; Liu et al. 1998; Hogg 1999). However, S-nitrosothiols will liberate free NO under certain circumstances, including the presence of copper (I) ions (Cu^+) or exposure to light (equation 7; Sexton et al. 1994; Gordge et al. 1995; Dicks and Williams 1996; Gorren et al. 1996; Singh et al. 1996).

$$R_1SNO + R_2S\text{-}N{=}O \longrightarrow R_1SH + R_2S\text{-}N{=}O \quad (6)$$

$$2RS\text{-}N{=}O \xrightarrow[\text{light}]{Cu^+} 2RSSR + 2NO \quad (7)$$

S-nitrosation of a range of cellular proteins is now considered to be one of a number of post-translational modifications that can alter protein function and these modifications are likely to be responsible for many of the cGMP-independent effects of NO (Li et al. 1997; Melino et al. 1997; Mohr et al. 1997).

1.2.3.3 Reaction of NO with Superoxide

A further critical reaction of NO in biological systems is that with the ROS, superoxide (O_2^-), resulting in the formation of peroxynitrite ($ONOO^-$: equation 8; Saran et al. 1990; Czapski and Goldstein 1995; Goldstein and Czapski 1995; Reiter et al. 2000). $ONOO^-$ rapidly forms peroxynitrous acid (ONOOH; equation 9), which then further decomposes to form NO_2 and hydroxyl radicals ($OH^{\bullet}$: equation 10; Wink et al. 1993; Butler et al. 1995).

$$NO + O_2^- \longrightarrow ONOO^- \quad (8)$$

$$ONOO^- + H+ \longrightarrow ONOOH \quad (9)$$

$$ONOOH \longrightarrow NO_2 + OH^{\bullet} \quad (10)$$

O_2^- is generated *in vivo* by enzymes including xanthine oxidase (Terada et al. 1991; White et al. 1996; Houston et al. 1999; Sawa et al. 2000; Spiekermann et al. 2003), the family of NADPH oxidases during host defence against invading pathogens (Mohazzab et al. 1994; Pagano et al. 1995; Somers et al. 2000; Forman and Torres 2001; Souza et al. 2002) and co-factor deficient NOS (Vasquez-Vivar et al. 1998; Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1999b). Additionally, O_2^- generated by the electron transport chain during respiration can 'leak' from the mitochondria (Zhang et al. 1998; Liu 1999; Staniek et al. 2002; Nohl et al. 2003). $ONOO^-$ is a powerful oxidising agent, which may be responsible for the cytotoxicity of high concentrations of NO in many cell types (Szabo 2003; Virag et al. 2003) including smooth muscle cells (Cao and Li 2004), endothelial cells (Chung et al. 2000; Hashida et al. 2000; Knepler et al. 2001) and macrophages (Tommasini et al. 2002). $ONOO^-$ is able to initiate lipid peroxidation (Radi et al. 1991; Graham et al. 1993; Rubbo et al. 1994; Leeuwenburgh et al. 1997a), DNA strand breaks (Szabo et al. 1996; Zingarelli et al. 1996; Guidarelli et al. 2000) and to induce various protein modifications including oxidation and nitration of, for example, tyrosine residues forming nitrotyrosine. The presence of nitrotyrosine is often taken to be the 'footprint' of protein damage by reactive nitrogen species such as $ONOO^-$ (van der

Vliet et al. 1996; Halliwell 1997; Reiter et al. 2000; Sawa et al. 2000; Virag et al. 2003; Radi 2004; Quijano et al. 2005).

Although generally accepted to be cytotoxic in most cells, there is some evidence that low concentrations of $ONOO^-$ can be beneficial. For example, $ONOO^-$ has been shown to induce vasodilatation (Davidson et al. 1997; Trakranrungsie and Will 2001; Li et al. 2004), inhibit platelet activation and aggregation (Brown et al. 1998; Low et al. 2002; Nowak and Wachowicz 2002) and, more recently, to induce apoptosis in human inflammatory cells – a process which is thought to be critical to the successful resolution of the inflammatory response (Blaylock et al. 1998; Taylor et al. 2004). The exact mechanism of these responses remains a source of debate; some studies suggest that $ONOO^-$ undergoes transnitrosation reactions with thiol groups and that the resultant S-nitrosothiols elicit the response (Mayer et al. 1995; van der Vliet et al. 1998). However, other studies suggest that S-nitrosothiols are not involved (Graves et al. 1998), and that $ONOO^-$ acts via an alternative mechanism, possibly by the nitrosation of tyrosine residues (Mondoro et al. 1997; Low et al. 2002; Nowak and Wachowicz 2002)

The reaction of NO with O_2^- is usually prevented *in vivo* by the battery of antioxidants that inactive ROS. For instance, the enzyme superoxide dismutase (SOD) removes O_2^- by catalysing its conversion to hydrogen peroxide (H_2O_2: equation 11), which is subsequently further inactivated by catalase (equation 12; Beckman and Koppenol 1996).

$$O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \quad (11)$$

$$O_2 + H_2O_2 \xrightarrow{Catalase} O_2 + H_2O \quad (12)$$

However, if the production of ROS is such that the antioxidant capacity of the cell is overcome, then any free NO present will combine rapidly with the ROS to form $ONOO^-$. This situation occurs in oxidative stress, which can be defined as an imbalance between oxidants and antioxidants in favour of the former. Overproduction of ROS and the resultant increase in oxidative stress has been implicated in the pathophysiology of endothelial dysfunction, which is thought to be a critical early event during atherogenesis (see section 1.4; Harrison et al. 2003; Schulz et al. 2004; Yokoyama 2004)

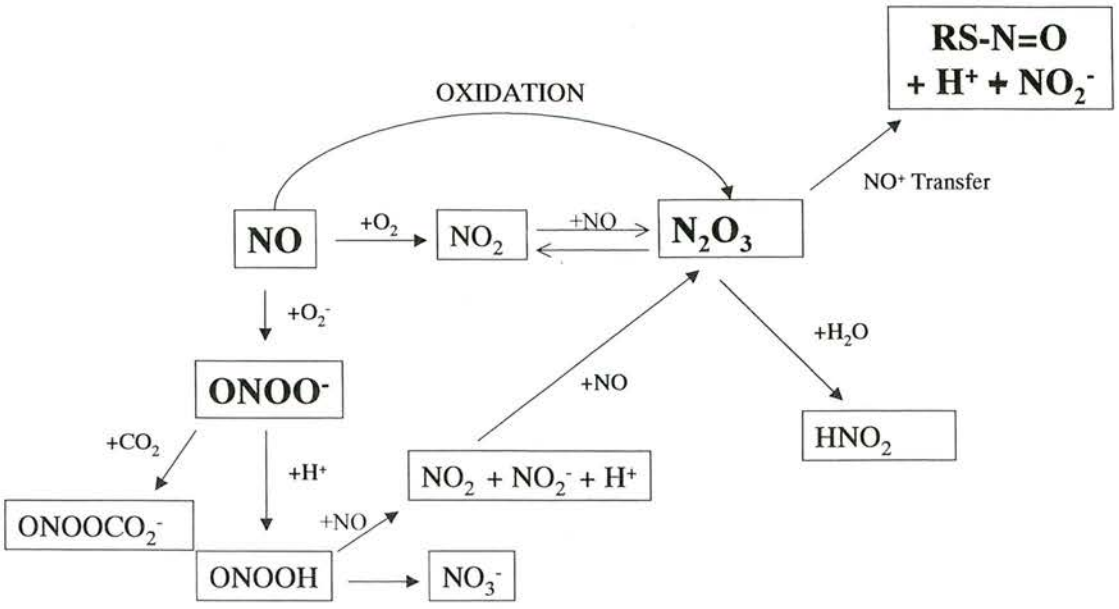

Figure 1.4 The Chemistry of NO

Nitric oxide (NO) can be oxidised to N_2O_3, which is able to transfer a nitrosonium ion (NO^+) to reduced cysteine residues, forming S-nitrosothiols (RS-N=O). NO will combine rapidly with superoxide ion (O_2^-) to form the oxidant species peroxynitrite ($ONOO^-$). $ONOO^-$ can react with H^+ to form peroxynitrous acid (ONOOH), which can subsequently combine with NO to generate N_2O_3.

1.2.4 iNOS-Derived NO

Although NO production is generally regarded to be beneficial, the high NO concentrations produced by iNOS in some pathologies can be detrimental. For example, high NO output from iNOS expressed in vascular smooth muscle cells (VSMCs) and endothelial cells in response to bacterial endotoxins produces global

vasodilatation and vascular collapse as a result of hyporesponsiveness to the vasoconstrictor, noradrenaline, during sepsis (Paya et al. 1995; Parratt 1998).

Subtrate-deficient iNOS has been demonstrated to generate O_2^- (Xia and Zweier 1997; Xia et al. 1998) and this O_2^- has been found to contribute to the vascular hyperactivity seen in a rat model of chronic heart failure (Miller et al. 2000). Furthermore, iNOS gene deficiency in the apolipoprotein E null (*Apo* $E^{-/-}$) mouse model of atherosclerosis results in reduced lesion development, due to decreased $ONOO^-$ production (Detmers et al. 2000; Kuhlencordt et al. 2001). These studies suggest that far from being beneficial, elevated levels of iNOS may contribute to, and exacerbate, the pathogenesis of these conditions. However, genetic deficiency of iNOS (*iNOS*$^{-/-}$) alone is not sufficient to reduce diet-induced atherosclerosis in mice wild type for the Apo-E gene (Niu et al. 2001), and administeration of L-Arg to replenish iNOS substrate levels negates the protective effects of iNOS gene deficieny in double knockout *Apo-*$E^{-/-}$*/iNOS*$^{-/-}$ mice (Chen et al. 2003). However, the possibility exists that the concentration of L-Arg administered may not have been sufficient to totally replenish substrate levels, or the enzyme may also have been deficient in additional co-factors, such as tetrahydrobiopterin. Alternatively, other sources of ROS may have been sufficient to counteract any additional NO production.

Inflammatory cells are potentially a major source of iNOS-derived NO as iNOS is up-regulated during non-selective host defence against invading pathogens and, therefore, may contribute substantially to the inflammatory response (Vane et al. 1994; Luss et al. 1996; Salvemini et al. 1996; Salvemini and Marino 1998).

However, the majority of studies examining the impact of iNOS on various conditions use primarily rodent models and although rodent inflammatory cells produce high levels of NO, this is not necessarily the case in human inflammatory cells. Despite the fact that human macrophages express iNOS messenger ribonucleic acid (mRNA) and protein, the output of NO in these cells is exceeding low or absent (Albina 1995; Weinberg et al. 1995; Thomassen and Kavuru 2001; Schneemann and Schoedon 2002)

Recently, it has been suggested that iNOS transcription may be crucial to the cytoprotective/cytotoxic balance of NO. Transcription of iNOS is controlled by the transcription factor, nuclear factor κB (NFκB), the activation of which can be regulated by NO. Hattori *et al* propose that NO suppresses, but $ONOO^-$ maintains NFκB activation (Hattori et al. 2004). Therefore, $ONOO^-$ is able to drive the production of iNOS-derived NO and, in cells that are already under oxidative stress, the NO generated will go on to form further $ONOO^-$, hence establishing a cycle of $ONOO^-$ production.

1.3 Apoptosis

Apoptosis, or programmed cell death, is a highly regulated, fundamental biological process governing cell survival. First described by Kerr *et al* in 1972 (Kerr et al. 1972), apoptosis differs from necrosis in that the cell is required to expend energy during apoptosis and the cell membrane remains intact, preventing the release of the cellular contents prior to removal by phagocytosis. A large number of pro- and anti-apoptotic mediators are contained within the cell, and it is the net

balance of these mediators that determines whether the cell undergoes apoptosis or remains viable.

Research interest in apoptosis has increased significantly in recent years following the discovery that many diseases are characterised by dysregulation of apoptotic pathways, including those with a chronic inflammatory component, such as atherosclerosis.

1.3.1 Characteristics and Pathways of Apoptosis

During the apoptotic process, cells undergo a number of characteristic morphological changes including membrane blebbing, chromatin condensation, nuclear fragmentation and cell shrinkage (Uren and Vaux 1996; Wyllie 1997). Cell surface changes, such as exposure of phosphatidylserine on the outer cell membrane, allow recognition by phagocytes (Fadok et al. 1992; Fadok et al. 2001; Hoffmann et al. 2001; Huynh et al. 2002). There are two principal apoptotic pathways: the death receptor pathway and the 'stress' or mitochondrial pathway.

1.3.1.1 Caspases

Caspase enzymes are aspartate-specific cysteine proteases which are the central effectors of apoptosis (Alnemri 1997; Miller 1997; Nicholson and Thornberry 1997). Regardless of whether apoptosis is initiated by the mitochondrial pathway or the death receptor pathway, caspases are the ultimate effectors of apoptosis. Caspases have the ability to auto-activate, or to be activated by other members of the caspase family, as part of an amplification cascade (Zimmermann et

al. 2001). Caspases-2, -8, -9, and -10, are generally classed as apoptotic initiators, whilst caspases-3, -6, and -7 are the apoptotic executioners. In non-apoptotic cells, caspases exist as inactive procaspases until initiation of apoptosis results in proteolytic cleavage and activation of the enzymes (Thornberry 1997; Kidd 1998). In addition, caspase activity is also controlled by natural caspase inhibitors present in cells, called inhibitors of apoptosis proteins (IAPs; Liston et al. 1996; Roy et al. 1997; Deveraux and Reed 1999; Deveraux et al. 1999).

The targets of caspases are the proteins involved in cell homeostatis and maintenance of the cell structure. For example, caspase-3 cleaves the inhibitor of caspase-activated deoxyribonuclease (iCAD) allowing the active enzyme, caspase-activated deoxyribonuclease (CAD), to cut the chromatin, resulting in DNA fragmentation (Liu et al. 1997; Enari et al. 1998; Sakahira et al. 1998). In addition to their role in DNA degradation, caspases are also responsible for the disassembly of the cytoskeleton through cleavage of structural proteins such as actin and fodrin, resulting in the dissociation of the cyoskeleton from the plasma membrane (Martin et al. 1995; Vanags et al. 1996). DNA degradation and disassembly of the cytoskeleton and nucleus result in the membrane blebbing, cell shrinkage and nuclear condensation characteristic of apoptosis (Thornberry 1997).

1.3.1.2 Death Receptor Pathway of Apoptosis

The death receptor pathway of apoptosis is triggered when ligands such as tumour necrosis α (TNF-α), Fas ligand (Fas-L), or TNF-related apoptosis-inducing ligand (TRAIL), activate their corresponding 'death receptors' – TNF receptor 1 (TNF-R1), the Fas receptor or the TRAIL receptor respectively. Death receptors are characterised by an extracellular region of cysteine-rich repeats, plus an intracellular amino acid sequence at the carboxy terminus of the receptor termed the 'death domain' (DD; Zimmermann et al. 2001). The DD is responsible for coupling the death receptor to a cascade of caspases, initiating apoptosis.

Ligation of a death receptor causes rapid formation of the death-inducing signalling complex (DISC). Adaptor proteins such as Fas-associated protein with death domain (FADD), or TNF-R1-associated protein with death domain (TRADD), are then recruited to the receptor death domain (Erickson 1997; Zimmermann et al. 2001; Algeciras-Schimnich et al. 2002). Adaptor proteins such as FADD also contain a death effector domain (DED) that is able to recruit procaspase-8 (Zimmermann et al. 2001; Boatright and Salvesen 2003). Procaspase-8 monomers undergo dimerisation to yield the active caspase-8 enzyme (Boatright and Salvesen 2003; Donepudi et al. 2003), which goes on to trigger the apoptotic caspase cascade via proteolytic cleavage of procaspase-3, -6 and -7 (Stennicke et al. 1998). Caspase-8 can also activate the mitochondrial pathway of apoptosis by causing Bid translocation to the mitochondria (see section 1.3.1.3; Zimmermann et al. 2001). Caspase-8 activation is additionally controlled by FLICE-like inhibitory protein (FLIP; caspase-8 was initially known as FLICE). FLIP can be both pro- and anti-

apoptotic depending on its concentration within the cell: at low levels of expression (close to those expected in a normal cell) FLIP enhances receptor-induced caspase-8 activation at the DISC, whilst at supraphysiological levels of expression FLIP can inhibit caspase-8 activation (Chang et al. 2002; Boatright and Salvesen 2003).

1.3.1.3 Mitochondrial Pathway of Apoptosis

Mitochondria play an essential role in apoptosis by releasing pro-apoptotic molecules, such as cytochrome *c*, into the cytoplasm which then go on to induce apoptosis (Green and Reed 1998). The mitochondrial pathway is broadly controlled by members of the Bcl-2 family of proteins (Kluck et al. 1997; Yang et al. 1997). Bcl-2 proteins can be both anti- and pro-apoptotic. Anti-apoptotic Bcl-2 proteins include (amongst others) Bcl-2, Bcl-X_L, and Bcl-w, whilst the pro-apoptotic members include Bid, Bad, Bim and Bik (Zimmermann et al. 2001). Anti-apoptotic Bcl-2 members promote cell survival by preventing cytochrome *c* release from the mitochondria (Kluck et al. 1997; Yang et al. 1997); pro-apoptotic members initiate cytochrome *c* release.

During apoptosis, the pro-apoptotic Bcl-2 proteins are activated and translocate to the mitochondria where they trigger the release of cytochrome c into the cytoplasm. Following cytochrome c release, the 'apoptosome', consisting of cytochrome *c*, apoptotic protease activating factor1 (Apaf-1) and procaspase-9, forms (Zou et al. 1999). The binding of Apaf-1 to procaspase-9 in the apoptosome results in activation of procaspase-9 (Rodriguez and Lazebnik 1999; Stennicke et al. 1999),

which then recruits and cleaves procaspase-3 forming activate caspase-3 to execute apoptotic death.

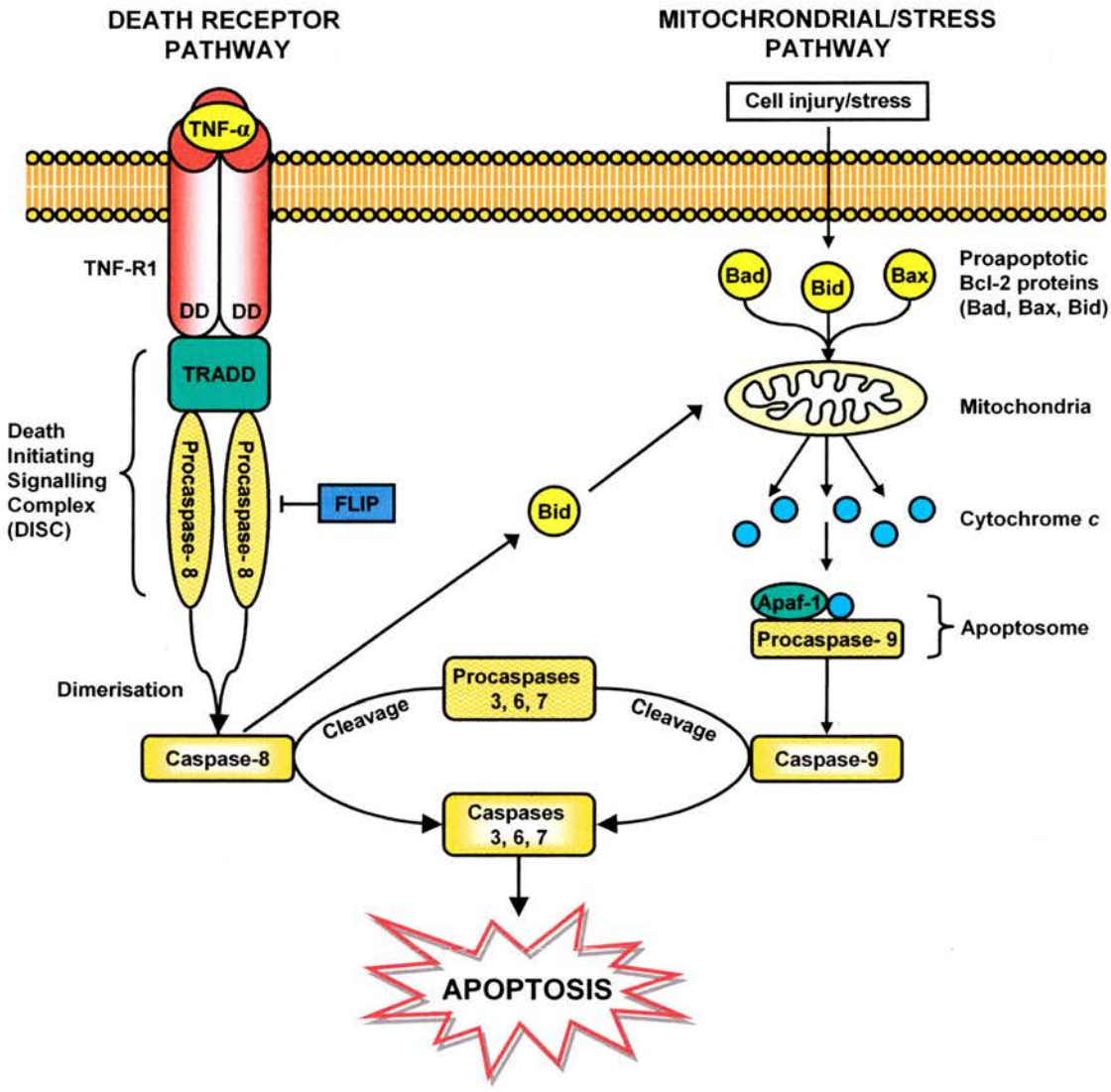

Figure 1.5 Schematic Representation of the Pathways of Apoptosis

Engagement of death receptors (eg TNFR-1) causes DISC formation and activation of caspase-8, which in turn activates the effector caspases-3,6 & 7 to trigger apoptosis. Additionally caspase-8 is able to initiate the mitchondrial pathway by causing translocation of the Bcl-2 protein, Bid, to the mitochondria. Pro-apoptotic Bcl-2 members cause cytochrome *c* release from the mitochondria. Cytochrome *c* combines with apoptotic protease activating factor1 (Apaf-1) and procaspase-9 to form the apoptosome, resulting in generation of active caspase-9. Caspase-9 activates caspases-3,6 & 7 to trigger apoptosis.

1.3.2 Inflammatory Cell Apoptosis

Because inflammatory cell apoptosis occurs without disruption of the cell membrane and ensuing release of histotoxic and pro-inflammatory mediators from the cytoplasm (Meagher et al. 1992; Stern et al. 1996; Lawrence et al. 2002), apoptosis represents a non-inflammatory mechanism for the removal of cells from a site of tissue damage, and hence, is critical to the successful resolution of the inflammatory response (Haslett 1997; Maderna and Godson 2003; Taylor et al. 2003; Rossi et al. 2004). Apoptotic cells are instantly recognised by phagocytes, such as macrophages, and removed from the site of inflammation (Savill et al. 1989; Fadok et al. 1992; Savill et al. 1993). Failure of inflammatory cells to undergo apoptosis, or failure of the subsequent phagocytic removal of apoptotic cells, for example, if the population of phagocytes is overwhelmed by the number of apoptotic cells, incomplete resolution of the inflammatory response can occur (figure 1.6). This happens because apoptotic cells remaining *in situ* undergo secondary necrosis, during which the cell membrane ruptures and the pro-inflammatory, histotoxic granule contents are released into the surrounding tissue, causing an exacerbation of the inflammatory response (Haslett 1997).

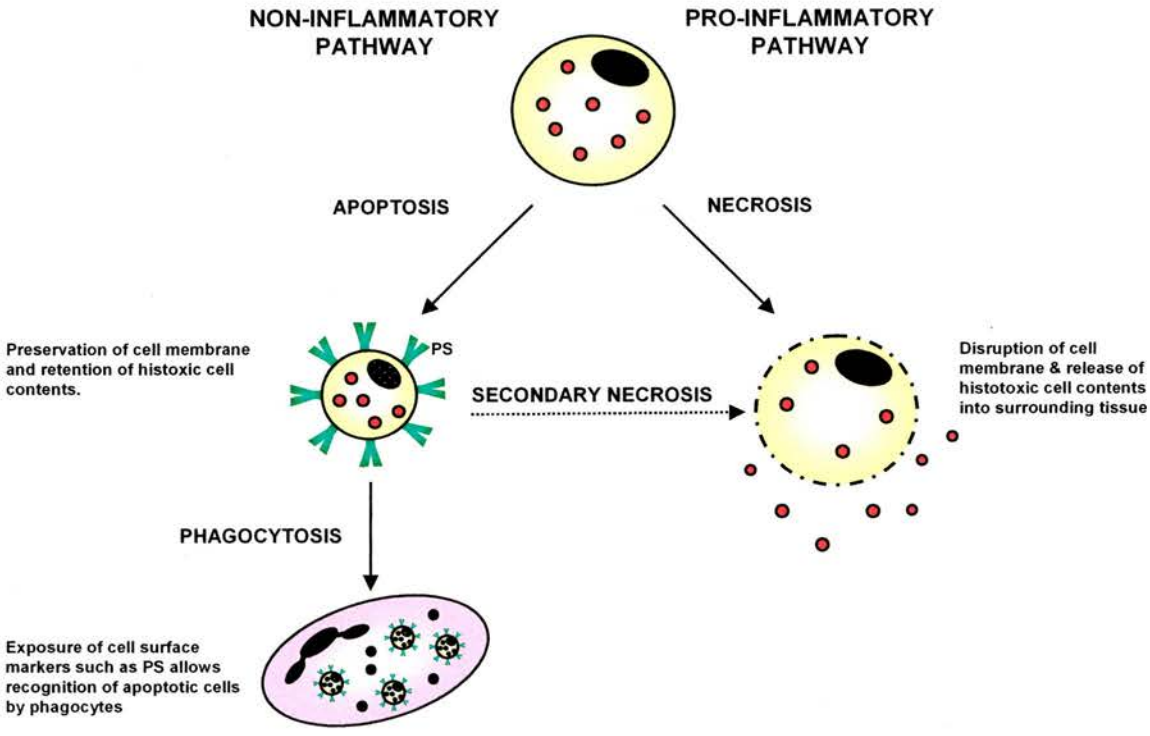

Figure 1.6 Non-Inflammatory and Pro-Inflammatory Fate of Inflammatory Cells

During apoptosis the cell membrane is maintained and the histoxic cell contents retained within the cell. Apoptotic cells are recognised by phagocytes and removed from a site of inflammation. Failure, or overwhelming of phagocytic clearance mechanisms leads to secondary necrosis. During necrosis the cell membrane is disrupted, causing release of the histotoxic cell contents into the surrounding tissue and resulting in an exacerbation of the inflammatory response.

1.3.3 NO as a Mediator of Apoptosis

NO can be both pro- and anti-apoptotic in many cell systems. As apoptosis involves manifold pathways and signalling cascades, it is likely there are multiple NO-dependent targets for the regulation of apoptosis, depending on the nature of the target cell. Current evidence suggests that lower NO concentrations produced

constitutively by eNOS and nNOS are cytoprotective via primarily cGMP-dependent mechanisms, whilst higher, supraphysiological concentrations generated in some pathologies by iNOS mediate apoptosis via mechanisms independent of cGMP (Nicotera et al. 1997). For example, high concentrations of either exogenous or endogenous iNOS-derived NO have been shown to induce apoptosis in various cell types including macrophages, endothelial cells and smooth muscle cells (Albina et al. 1993; Sarih et al. 1993; Muhl et al. 1996; Bennett and Boyle 1998), and this has been demonstrated to occur independently of cGMP signalling (Wang et al. 1999). The precise downstream effectors of NO-induced apoptosis remain to be elucidated, but there is some evidence to suggest that NO causes a profound down-regulation of IAP proteins (Manderscheid et al. 2001). However, this effect is cell type specific, occurring in macrophages but not mesangial cells. Alternatively, NO may act to enhance, or sensitise cells to Fas/Fas-L mediated apoptosis (Garban and Bonavida 1999; Boyle et al. 2002).

Conversely, pre-treatment of RAW 264.7 cells (a mouse macrophage cell line) with relatively low concentrations of exogenous NO, delivered by synthetic NO donor compounds, confers cGMP-dependent protection against cell death upon subsequent exposure to concentrations of NO which would normally be sufficient to be cytotoxic (von Knethen et al. 1999; Yoshioka et al. 2003). Similarly, endogenous eNOS and iNOS-derived NO inhibits apoptosis in various cell types, possibly by regulating levels of death receptor ligands such as TNF-α and Fas-L (Furuke et al. 1999; Hatano et al. 2001). S-Nitrosothiols have also been demonstrated to decrease

apoptosis by directly inhibiting caspase-3 activation via S-nitrosation of thiol groups on the enzyme (Kim et al. 1997; Mohr et al. 1997).

The dual effect of NO in apoptosis may be attributed to the chemistry of NO and the ease with which it forms various NO-related species (see section 1.2.3). A critical reaction of NO *in vivo* is that with O_2^-, resulting in the formation of $ONOO^-$. The precise role of $ONOO^-$ in inflammatory cell apoptosis remains to be elucidated. There is some evidence to suggest that at high concentrations (100–300 μM), $ONOO^-$ induces apoptosis in RAW 264.7 cells (Sandoval et al. 1997), whilst at lower concentration (30–50 μM) it may have a protective effect against lipopolysaccharide (LPS) and interferon (IFN)-γ-induced apoptosis in these cells (Scivittaro et al. 1997). Brockhaus *et al* have demonstrated that over-expression of copper/zinc SOD (CuZnSOD) can protect RAW 264.7 cells against apoptosis initiated by NO, either exogenous or iNOS-derived, suggesting $ONOO^-$ may be the mediator of NO-induced apoptosis (Brockhaus and Brune 1999). Similarly, delivery of exogenous $ONOO^-$ has recently been shown to induce apoptosis in human neutrophils (Taylor et al. 2004).

Whilst endogenous macrophage iNOS-derived NO has been shown to induce apoptosis in animal models, this is not necessarily the case in human macrophages, where NO production is exceedingly low or absent (Albina 1995; Weinberg et al. 1995; Thomassen and Kavuru 2001; Schneemann and Schoedon 2002). However, despite this reduced capability to produce endogenous NO, human macrophages do undergo apoptosis in response to exogenous NO and NO-related species. For example, the NO donors S-nitrosoglutathione (GSNO) and spermine

diazeniumdiolate (SPER/NO) induce apoptosis in primary human monocytes-derived macrophages (von Knethen et al. 1999).

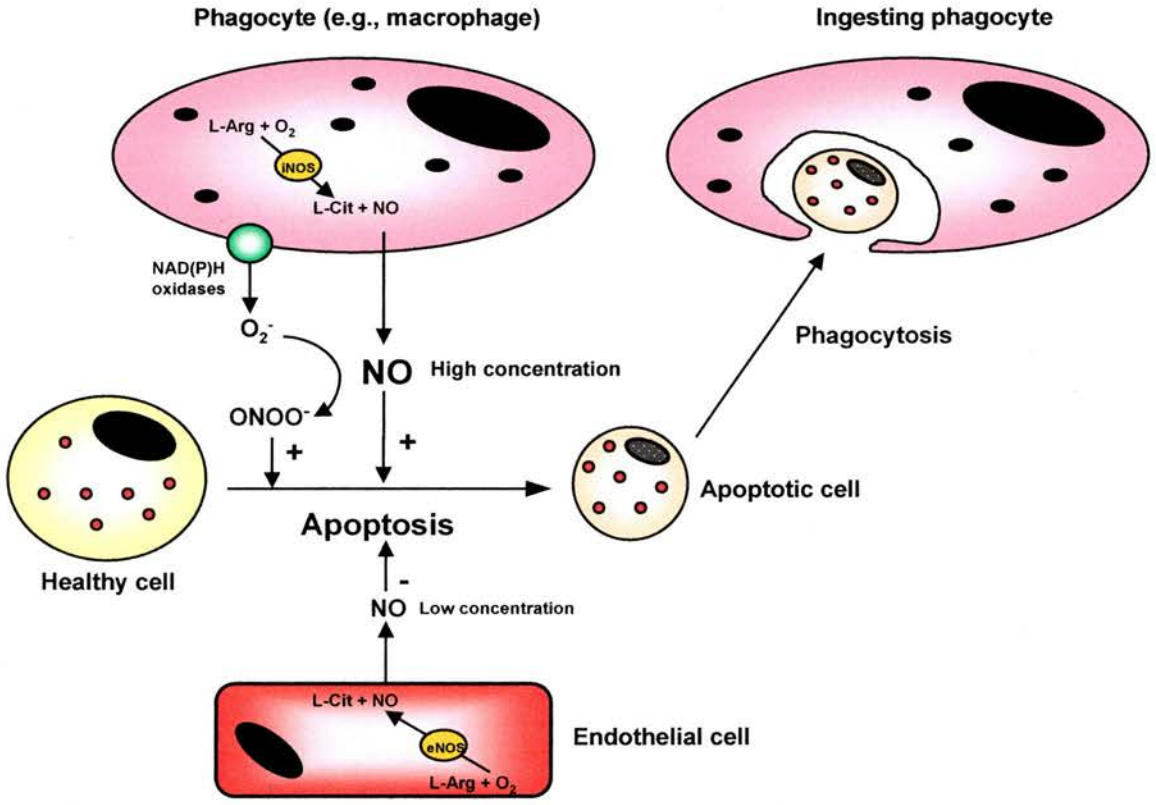

Figure 1.7: **The Pro- and Anti-Apoptotic Actions of NO**.

High concentrations of NO synthesised by iNOS in phagocytes, such as macrophages, induce apoptosis in neighbouring cells. In addition, apoptosis can also be induced by $ONOO^-$ generated as O_2^- produced by phagocytes reacts with NO. Apoptotic cells are subsequently recognised and ingested by phagocytes, thus aiding the resolution of inflammation. Conversely, low concentrations of NO produced constitutively by eNOS in endothelial cells can inhibit apoptosis.

1.4 Atherosclerosis

Atherosclerosis is characterised by the formation of lipid-rich plaques in the sub-endothelial space of large conduit blood vessels (Badimon et al. 1993; Ross 1993; Ross 1999a; Ludewig et al. 2002). These plaques, which are the hallmark of the disease, are usually eccentric (occupying one side of the vessel; figure 1.8) and consist of a necrotic core of lipid-laden inflammatory cells encapsulated by a fibrous, collagen-rich cap made up of vascular smooth muscle cells (VSMC) and extracellular matrix (Ross 1993; Davies 1997; Ross 1999a). Advanced lesions may also be vasculised by new blood vessels (O'Brien et al. 1994; Kumamoto et al. 1995) and are calcified (Proudfoot et al. 1998; Abedin et al. 2004; Speer and Giachelli 2004; Bobryshev 2005).

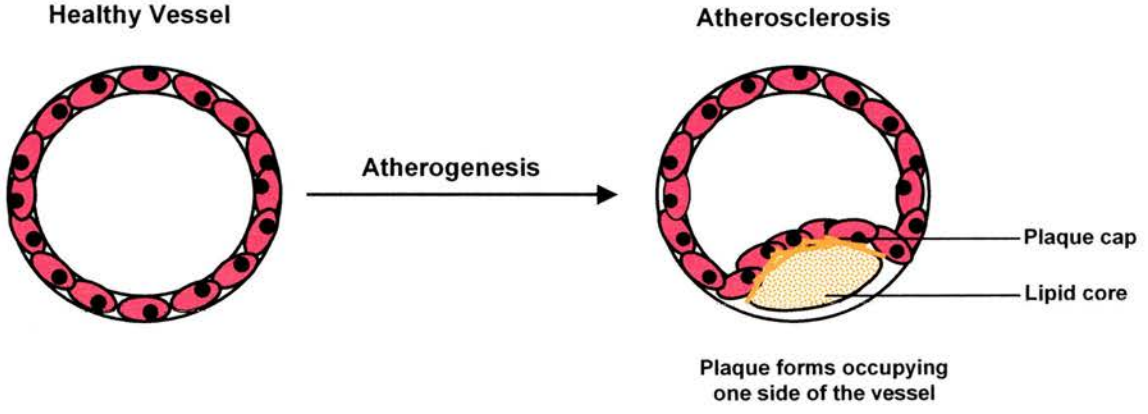

Figure 1.8 Schematic Representation of the Eccentric Position of Atherosclerotic Plaques

Atherosclerotic plaques form in the vessel wall and are usually eccentric – occupying one side of the vessel.

1.4.1 Atherogenesis

Despite decades of arduous research efforts in both humans and animal models of atherosclerosis, the underlying causes of atherogenesis remain largely unknown. Atherosclerotic lesions are known to be initiated early in life: in 1953 Enos *et al* described advanced lesions in the coronary arteries of young soldiers killed in the Korean War, whose average age at the time of death was just 23 (Enos et al. 1953), and the earliest recognisable lesions of atherosclerosis, non-stenotic 'fatty streaks', have been observed in children, including neonates (McGill et al. 2000).

1.4.1.1Endothelial Dysfunction in Atherosclerosis

In healthy vessels, endothelium-derived NO contributes not only to control of vascular tone but also to the maintenance of vessel wall integrity and to inhibition of platelet and leucocyte activation (Radomski et al. 1987a; Radomski et al. 1987b; Gauthier et al. 1994; Kanwar and Kubes 1995). A critical early event during atherogenesis is believed to be endothelial cell injury and damage, resulting in endothelial dysfunction. One of the major consequences of endothelial dysfunction is a decrease in the net bioavailability of NO. This is seen in conditions predisposing to atherosclerosis such as hypercholesterolaemia, diabetes and cigarette smoking. Such conditions are associated with endothelial dysfunction, which is usually assessed in terms of impaired of endothelium-dependent vasomotion that can be reversed by delivery of exogenous NO (Osborne et al. 1989; Chowienczyk et al.

1992; Kim et al. 1994; Zeiher et al. 1995b; Cooper and Heagerty 1998; Cosentino and Luscher 1998; Poredos et al. 1999; De Vriese et al. 2000; Esen et al. 2004).

Endothelial dysfunction can result from oxidative stress and the retention in the vessel wall of pathogenic oxidation-modified low density lipoproteins (ox-LDL - see section 1.4.2.1; Simon et al. 1990; Tanner et al. 1991; Buckley et al. 1996; Mougenot et al. 1997). Additionally, physical damage to endothelial cells can occur as a result of abnormal shear stress within the vessel. Atherosclerotic plaques tend to form in areas of the arterial tree exposed to particularly turbulent blood flow, such as bifurcation points, or in areas exposed to particularly low shear stress (Ku et al. 1985; Salzar et al. 1995; Thubrikar and Robicsek 1995; Gnasso et al. 1997; Jiang et al. 2000). Conversely, plaque initiation and progression is significantly decreased in regions of laminar flow. This is thought to be because laminar flow is a stimulus for transcription of atheroprotective genes, such as the genes for eNOS and SOD (Busse and Fleming 1998; Traub and Berk 1998; Silacci et al. 2000; Berk et al. 2001), and because shear stress is a major activator of NOS (Gallis et al. 1999; Fisslthaler et al. 2000; Busse and Fleming 2003; Fleming and Busse 2003)

1.4.1.2 Inflammation in Atherosclerosis

It is now broadly accepted that there is an inflammatory component to atherosclerosis (Badimon et al. 1993; Vogel 1997; Ross 1999a; Ross 1999b; Berk et al. 2001; Libby 2002; Robbins and Topol 2002). This was first described in 1989 when Hanson *et al* detected activated T-lymphocytes in human atherosclerotic plaques excised from carotid endarterectomy specimens and proposed a mechanism

for the involvement of the immune system in atherosclerosis (Hansson et al. 1989a; Hansson et al. 1989b). Since then, this hypothesis has been widely accepted and atherosclerosis is now considered to be the consequence of a chronic low-grade inflammatory process which, over time, comes to constitute the disease process itself (Badimon et al. 1993; Vogel 1997; Ross 1999a; Ross 1999b; Berk et al. 2001; Libby 2002; Robbins and Topol 2002).

The inflammatory response, widely acknowledged to be initiated by the retention of pathogenic lipids in the vessel wall and consequent endothelial injury, is characterised by the expression of numerous cytokines and adhesion molecules for mononuclear leucocytes on the surface of activated endothelial cells (Ross 1999a; Ross 1999b; Libby 2002). These molecules, including integrins, immunoglobulins, and selectins and their ligands, are responsible for the recruitment of leucocytes to the site of inflammation through a series of events involving leucocyte rolling along the endothelial surface, firm leucocyte adhesion and activation at the site of inflammation, followed by diapedesis through the endothelium to the vessel intima (Lefer and Granger 1999; Huo and Ley 2001). A variety of studies in both atherosclerosis-prone mice and human excised arteries containing plaques have demonstrated that the endothelium in lesion-prone areas, or that overlying pre-existing plaques, expresses many leucocyte adhesion molecules, which include P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1; Davies et al. 1993; Ramos et al. 1999; Collins et al. 2000; Dong et al. 2000; Huo et al. 2000; Huo et al. 2001). Of these, VCAM-1, a member of the cytokine-inducible Ig superfamily which bind leucocyte integrins, has

been identified as playing a critical role in early atherogenesis and one which is distinct from the structurally and functionally similar ICAM-1 (Cybulsky et al. 2001). The importance of leucocyte adhesion and migration during atherogenesis has been further illustrated in studies in which the gene for various adhesion molecules or chemokines have been deleted in mice already genetically susceptible to atheroma, such as the apolipoprotein E null (*Apo* $E^{-/-}$), or the LDL receptor null ($LDLr^{-/-}$) mouse models of the disease. In these studies, the double knockout mice have markedly reduced lesion area and show greatly reduced monocyte accumulation within the plaque compared to their single knockout *Apo* $E^{-/-}$ or LDL$r^{-/-}$ controls (Boisvert et al. 1998; Boring et al. 1998; Gu et al. 1998; Huo and Ley 2001).

Once resident in the intima, monocytes differentiate into macrophages under the influence of various colony stimulating factors, such as macrophage colony-stimulating factor (M-CSF; Takahashi et al. 2002). M-CSF expression is elevated in both human and animal plaques and, when deleted in atherosclerosis-prone mice, lesion development is greatly retarded due to decreased macrophage accumulation (Clinton et al. 1992; Rosenfeld et al. 1992; Smith et al. 1995). Following differentiation, macrophages express scavenger receptors facilitating the internalisation of ox-LDL (Henriksen et al. 1981; Parthasarathy et al. 1986; Haberland et al. 1992; Parthasarathy et al. 1992; Hazell and Stocker 1993). Accumulation of ox-LDL is allowed to continue unchecked because, unlike the native LDL receptor, scavenger receptors are not down-regulated once the cell is cholesterol-replete (Brown and Goldstein 1983). This results in the formation of cytosolic droplets of cholesterol and cholesterol esters within macrophages. In this

lipid-laden condition, macrophages are known as foam cells and it is an accumulation of foam cells which characterises the earliest recognisable atherosclerotic lesions, known as 'fatty streaks' (Schwartz et al. 1991; Ross 1993). However, scavenger receptors can also regulate cholesterol efflux from the cell and, therefore, foam cell formation is a dynamic process and the net result of total cholesterol flux within the cell (Schwartz et al. 1991).

1.4.1.2.1 NO as an Anti-Inflammatory Agent in Atherosclerosis

Following the establishment of inflammation as a critical process during initiation, progression and complication of atherosclerotic plaques (Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002; Robbins and Topol 2002), many studies have demonstrated an anti-inflammatory role for NO. Pharmacological inhibition of NOS to limit NO production has been demonstrated to increase monocyte adhesion and infiltration into vessels in animal models of atherosclerosis, whilst administration of L-Arg or NOS gene transfer to boost NO production decreases inflammatory cell adhesion (Tsao et al. 1994; Tomita et al. 1998; Qian et al. 1999). Similarly, leucocyte rolling and leucocyte-endothelial interactions are increased in eNOS and nNOS deficient mice (Lefer et al. 1999). Delivery of exogenous NO to human endothelial cell cultures results in a decreased expression of adhesion molecules, such as VCAM-1, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), which are thought to be critical for the migration of monocytes into the intima at sites of atherosclerotic lesion formation (De Caterina et al. 1995; Zeiher et al. 1995a; Tomita et al. 1998).

1.4.2 Atherosclerotic Plaque Progression

Once the fatty streak has been established, the plaque evolves and develops into a complicated lesion consisting of a necrotic core of lipid-laden inflammatory cells encapsulated by a layer of VSMC, forming a fibrous cap over the lesion surface (Ross 1993; Davies 1997; Ross 1999a). Plaque growth occurs as the ox-LDL accumulated in macrophage-derived foam cells is released into the surrounding tissue following necrosis of foam cells and this causes further endothelial damage (Simon et al. 1990; Tanner et al. 1991; Buckley et al. 1996; Mougenot et al. 1997) and is chemoattractant for additional inflammatory cell recruitment (Quinn et al. 1987; McMurray et al. 1993; Ruan et al. 1996; Wang et al. 1997a; Wang et al. 1997b). Hence, a perpetual cycle of endothelial damage, monocyte recruitment and peroxidation and accumulation of lipids is established.

1.4.2.1 Lipid Peroxidation in Atherosclerosis

Peroxidation of lipids was proposed as a key step in atherogenesis following the observation that oxidative modification of LDL by cells, or by oxidising species generated *in vivo*, could cause LDL to become cytotoxic (Hessler et al. 1983; Morel et al. 1983; Morel et al. 1984). Internalisation and accumulation of lipids in the form of cholesterol and cholesterol esters is a central feature of atherosclerosis, with lipid-laden cells eventually becoming necrotic and releasing their contents to form the plaque core. However, native lipids do not accumulate in cells because the native LDL receptor is down-regulated once the cell becomes cholesterol-replete (Brown and Goldstein 1983). Lipids only accumulate once they have been modified,

rendering them recognisable by scavenger receptors which are not down-regulated by cholesterol loading in the cell (Henriksen et al. 1981; Parthasarathy et al. 1986; Haberland et al. 1992; Parthasarathy et al. 1992; Hazell and Stocker 1993). The LDL particle consists of an apolar core of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids, unesterified cholesterol and the protein molecule apolipoprotein B 100 (Orlova et al. 1999; Rubbo and O'Donnell 2005). In addition, lipophilic antioxidants, including α-tocopherol (vitamin E) and carotenoids are also present in the LDL particle (Rubbo and O'Donnell 2005). Modification of LDL can occur by acetylation, and, possibly most importantly in the context of atherosclerosis, oxidation. Polyunsaturated fatty acid residues (those that contain more than one carbon-carbon double bond) in the LDL particle are more susceptible to oxidation, as the carbon-hydrogen bond adjacent to the double bond is weaker and therefore, the hydrogen atom is easier to abstract from the bis-allylic methylene ($-CH_2-$) group. Hydrogen atom abstraction results in the formation of a lipid peroxyl radical (Holvoet and Collen 1994; Maxwell and Lip 1997). The peroxyl radical formed is able to abstract a hydrogen atom from an adjacent unsaturated fatty acid producing a second peroxyl radical which goes on to oxidise surrounding fatty acids. Thus, a self-propagating chain reaction is established. This sequence of oxidation events can be terminated by 'chain breaking' anti-oxidants, which react with reactive radicals to form unreactive radicals. Such compounds, including glutathione, vitamin E, vitamin C and ubiquinol-10, are electron rich compounds, which are sacrificed (oxidised) when the cell is under oxidative stress (Carr et al. 2000b). Additionally, enzymes such as SOD, catalase, and glutatione peroxidase remove damaging oxidant

species from the cell. Only when oxidative stress within a cell has overcome the intracellular anti-oxidant defence capacity, will damaging oxidation occur. Such an event might occur as a result of increased generation of oxidants or depletion or down-regulation of antioxidant defences, as has been demonstrated recently in a study showing that glutathione synthesis is depleted in advance of lipid peroxidation and detectable atheroma in *ApoE*$^{-/-}$ mice (Biswas et al. 2005).

The initiators of lipid peroxidation *in vivo* are still not fully established, but possible oxidising candidates include copper (and other transition metal) ions, either free or bound in prosthetic groups of proteins, for example the haem group in proteins such as haemoglobin (Heinecke et al. 1984; Heinecke 1997a). Metal ions are able to react with H_2O_2 in the Fenton Reaction to generate powerful oxidising $OH^{\bullet}$ radicals (Valko et al. 2005). However, given that these ions are present under normal physiological conditions, perhaps more likely is decreased anti-oxidant capacity within the cell, occurring in combination with the generation of powerful oxidising free radical species present *in vivo* (Heinecke 1997a; Maxwell and Lip 1997). There are numerous sources of oxidising free radical species present in the vessel wall including xanthine oxidase (Terada et al. 1991; White et al. 1996; Houston et al. 1999; Sawa et al. 2000; Spiekermann et al. 2003), co-factor deficient NOS (Vasquez-Vivar et al. 1998; Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1999b), and the mitochondrial electron transport chain (Zhang et al. 1998; Liu 1999; Staniek et al. 2002; Nohl et al. 2003). Additionally, activated inflammatory cells present in the vessel wall express enzymes capable of producing oxidising species usually associated with host defence against invading pathogens (for example, the

family of NADPH oxidases and myloperoxidase (MPO; Klebanoff 1980; Mohazzab et al. 1994; Pagano et al. 1995; Somers et al. 2000; Forman and Torres 2001; Souza et al. 2002)). MPO, a member of the haem peroxidase superfamily generates various species implicated in the oxidation of lipids, including H_2O_2, hyperchlorous acid (HOCl), and the products of L-tyrosine oxidation - tyrosyl radical and p-hydroxyphenylacetaldehyde (pHA; Savenkova et al. 1994; Heinecke 1997b; Yang et al. 1999; Carr et al. 2000a; Heller et al. 2000; Jerlich et al. 2000; Podrez et al. 2000). The presence of the MPO enzyme has been demonstrated in human atherosclerotic lesions (Daugherty et al. 1994), and specific markers of MPO-mediated lipid modification, for example 3-chlorotyrosine and dityrosine, have been isolated from both animal and human atherosclerotic plaques, suggesting that this may be the most physiological relevant mechanism of lipid peroxidation during atherogenesis (Hazen and Heinecke 1997; Leeuwenburgh et al. 1997b; Heinecke 1999; Hazen et al. 2000; Heller et al. 2000).

1.4.2.1.1 Pro-Oxidant Role of NO in Lipid Peroxidation

The role of NO in lipid peroxidation is complex due to its ability to form various NO-related species. NO can be both pro- and anti-oxidant towards lipids, depending on the redox balance of the microenvironment. NO alone is not a strong enough oxidising agent to initiate the peroxidation chain reaction (Darley-Usmar et al. 1992; Rubbo et al. 1994; Hogg and Kalyanaraman 1999). However, mass spectrometric studies have revealed significantly elevated levels of nitrotyrosine (a marker for protein damage by reactive nitrogen species (van der Vliet et al. 1996;

Halliwell 1997)) in LDL recovered from human atherosclerotic aortae compared to LDL isolated from plasma of healthy donors (Leeuwenburgh et al. 1997a). NO in combination with O_2^- results in the formation of $ONOO^-$ (see section 1.2.3.3) which is a powerful oxidising agent and able to initiate LDL oxidation (Radi et al. 1991; Darley-Usmar et al. 1992; Hogg et al. 1993a; Hogg et al. 1993b; Leeuwenburgh et al. 1997a). Furthermore, this $ONOO^-$-mediated modification leads to recognition by scavenger receptors and rapid accumulation in macrophages (Graham et al. 1993). Graham *et al* propose that $ONOO^-$ may act to modify lipids by altering the protein, lipid and anti-oxidant composition of LDL, most notably, by depleting the anti-oxidant vitamin E content of the particle via the conversion of α-tocopherol to α-tocopherol quinone (Graham et al. 1993). Reactive nitrogen species are also involved in MPO-mediated lipid oxidation (Byun et al. 1999; Hazen et al. 1999; Schmitt et al. 1999), because nitrite (NO_2^-) acts as a co-substrate for MPO and will also react with HOCl to generate nitrating and chlorinating species (Eiserich et al. 1996; van der Vliet et al. 1997; Eiserich et al. 1998; Sampson et al. 1998). MPO-generated reactive nitrogen species have been demonstrated to convert LDL into an atherogenic form (Podrez et al. 1999) and lipids damaged by reactive nitrogen species have been isolated from human atherosclerotic intima (Pennathur et al. 2004).

1.4.2.1.2 Anti-oxidant Role of NO in Lipid Peroxidation

In addition to the pro-oxidant characteristics of NO-related species, there is some evidence to suggest that NO is able protect against LDL oxidation, providing the concentration is sufficient to counteract any other oxidising agents present in the

cell. Relatively high NO concentrations (12 μM) generated by murine cytokine-stimulated macrophages have been demonstrated to inhibit macrophage-driven lipid peroxidation (Yates et al. 1992). Similarly, continuous NO release from NO donor drugs inhibits LDL oxidation, possibly because NO is able to act as a chain breaking anti-oxidant by reacting with lipid radicals such as alkyl (L·), alkoxyl (LO·) and peroxyl (LOO·) radicals (Hogg et al. 1993c; Padmaja and Huie 1993; O'Donnell et al. 1997; Hogg and Kalyanaraman 1999; O'Donnell and Freeman 2001).

1.4.2.2 The Plaque Cap

VSMC proliferation is an important component of vessel wall remodelling in response to injury during atherosclerosis. Activated VSMCs undergo hypertrophy and/or hyperplasia during the atherosclerotic process to form the plaque cap, which also contains endothelial cells and fibroblasts (Ross 1993). During this process, VSMCs undergo phenotyptic alteration from the adult vascular contractile phenotype, to a synthetic phenotype usually seen in developing vessels, and the VSMCs of the plaque cap are now considered to have a distinct phenotype (Bonin et al. 1999; de Vries et al. 2000; Blindt et al. 2002; Zhang et al. 2002; Mulvihill et al. 2004). However, characterisation of VSMCs into only two distinct phenotypes is a vast over-simplification. A wide spectrum of diverse, intermediary, phenotypes with differential gene expression, exist under different physiological and pathophysiological conditions, without a clear distinction between phenotypes. This is especially true when VSMCs are undergoing phenotypic transition and there may be several sub-populations of VSMCs present within the plaque at any given time

(Owens et al. 2004). In a synthetic phenotype, VSMCs have the ability to proliferate and produce connective tissue and extracellular matrix macromolecules including elastin and collagen (Newby and George 1996). These molecules form a mesh over the fatty streak and, following calcification, form the fibrous cap over the plaque, encapsulating the highly thrombogenic lipid core and maintaining a barrier between the plaque contents and the circulation (Badimon et al. 1993; Davies 1997).

There is currently some debate as to the origins of VSMCs present in the cap. Initially they were thought to migrate from the media into the intima, however, recently it has been proposed that there may be bone marrow-derived smooth muscle progenitor cells circulating in the blood which are then recruited to the site of vessel damage (Hillebrands et al. 2001; Sata et al. 2002; Simper et al. 2002; Deb et al. 2004).

1.4.2.2.1 The Role of NO in VSMC Proliferation

Numerous studies have demonstrated that NO is able to inhibit VSMC proliferation (Garg and Hassid 1989; Kariya et al. 1989; Nakaki et al. 1990; Assender et al. 1992; Newby et al. 1992; Mooradian et al. 1995; Jeremy et al. 1999). The exact mechanism of this inhibition remains to be elucidated, with some authors reporting that the anti-proliferative of NO are cGMP-dependent (Garg and Hassid 1989), whilst others suggest cGMP-independent inhibition of the enzymes arginase and ornithine decarboxylase is involved (Ignarro et al. 2001).

The ability of NO to inhibit VSMC proliferation has led to the hypothesis that reduced NO bioavailability occurring as a result of endothelial dysfunction in

atherosclerosis may contribute to the formation of the plaque cap. A number of studies have reported that eNOS gene therapy inhibits luminal narrowing following vascular injury by preventing VSMC proliferation (von der Leyen et al. 1995; Tzeng et al. 1996; Janssens et al. 1998). However, these studies are not strictly a model of atherosclerosis, and the presence of the cap may be a protective response, preventing plaque rupture (see section 1.4.3).

1.4.3 Atherosclerotic Plaque Rupture

Although atherosclerotic lesions may be widespread by middle age, the vast majority remain sub-clinical with only a small minority of plaques becoming symptomatic (Rudd et al. 2005). The physical presence of a plaque within an arterial wall may impinge on the vessel lumen causing partial occlusion of the vessel. Such an occlusion may be sufficient to cause substantial blood flow restriction and tissue ischaemia, which, if occurring in the coronary vessels, results in chronic stable angina pectoris. However, arteries are dynamic rather than static and can remodel to compensate for this, allowing a vessel to accommodate an expanding plaque without a significant decrease in lumen diameter (Glagov et al. 1987). Thus, large atherosclerotic lesions may be, and often are, considered 'stable' and remain asymptomatic. Of far greater clinical significance are unstable, or ruptured plaques. Plaque instability occurs if the cap is subject to mechanical breakdown or erosion, increasing the propensity of a plaque to rupture.

In the stable plaque, the VSMC cap serves as a barrier to ensure the highly thrombogenic contents of the plaque core are separated from the circulation. When

the cap is compromised and the thrombogenic contents of the core are exposed to the circulation, platelets are rapidly recruited and activated, and a thrombus forms. Plaque rupture can occur several times and remain subclinical with the VSMC cap reforming, or healing, over the top of the thrombus which becomes incorporated into the growing lesion, resulting in a layering, or 'onion skin', effect within the plaque (Mann and Davies 1999). However, if the resulting thrombus is sufficiently large, it can further occlude the vessel *in situ* leading to the clinical symptoms of angina pectoris. Alternatively, if the thrombus detaches form the plaque surface, the resulting embolus can occlude smaller vessels downstream, leading to the acute cardiovascular syndromes, such as myocardial infarction and stroke (Davies 1995; Schroeder and Falk 1995; Dalager-Pedersen et al. 1998; Gutstein and Fuster 1999; Zhou et al. 1999; Corti and Badimon 2002; Mitra et al. 2004).

The determinants of plaque vulnerability to destabilisation and rupture have yet to be fully identified, but a growing body of evidence is emerging that points to a critical role for both the thickness of the VSMC layer overlaying the core (Leskinen et al. 2003) and to inflammatory processes occurring within the plaque (Libby 2002; Robbins and Topol 2002; Lombardo et al. 2004). For example, a plaque containing a large lipid pool, with a high inflammatory cell content and a thin fibrous cap containing relatively few VSMCs is more prone to rupture than a plaque with a lower inflammatory cell content and a thicker cap, containing a relatively high VSMC density (Davies 1996; Felton et al. 1997).

Given that plaque rupture is now considered the critical determinant of acute cardiovascular syndromes (Bonin et al. 1999; de Vries et al. 2000; Blindt et al. 2002;

Zhang et al. 2002; Mitra et al. 2004; Mulvihill et al. 2004), a clinical need exists to identify those individuals with potentially unstable plaques who are at high risk of cardiovascular events (Stefanadis et al. 2003; Rudd et al. 2005).

1.4.3.1 The Role of Matrix Metalloproteinases in Plaque Rupture

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of cleaving components of the extracellular matrix including collagen and elastin (Jones et al. 2003). Since their discovery in 1962, over 66 MMPs, including at least 20 in humans, have been identified (Ye 2000). The presence of elevated levels and activity of MMPs within atherosclerotic plaques, particularly at the shoulder region of the plaque cap (Henney et al. 1991; Galis et al. 1994; Halpert et al. 1996; Johnson et al. 1998), coupled with the observation that patients with acute coronary syndromes have elevated levels of MMP-9 compared to patients suffering stable angina (Nikkari et al. 1995; Sukhova et al. 1999; Inokubo et al. 2001; Uzui et al. 2002; Blankenberg et al. 2003), has led to speculation that MMPs might be involved in plaque rupture. The role of MMPs in atherosclerosis is complex due to the large number of isozymes with overlapping specificities. Numerous studies have suggested a role for various MMPs, particularly MMP-2 and MMP-9, in plaque instability (Sapienza et al. 2004; Kong et al. 2005; Tziakas et al. 2005), and genetic deficiency of MMP-9 protects $ApoE^{-/-}$ mice against atherosclerotic media destruction (Luttun et al. 2004). Furthermore, activated macrophages have been demonstrated to induce collagen breakdown in the fibrous cap of atherosclerotic plaques via activation of MMPs (Shah et al. 1995). However,

a recent study comparing divergent effects of various MMPs on plaque stability, found that whilst MMP-12 may act as a destructive protease promoting plaque instability, MMP-9 and MMP-3 have a protective role in reducing thrombus size and promoting smooth muscle-directed healing after plaque rupture (Johnson et al. 2005). The authors suggest that elevated MMP-9 levels are a consequence, rather than cause, of plaque rupture and that increased MMP-9 expression results in increased stability of the newly re-formed cap (Johnson et al. 2005).

1.4.3.2 The Role of Apoptosis in Plaque Rupture

Apoptosis of several cell types, notably monocytes/macrophages, endothelial cells and VSMCs, may play a pivotal role in atherosclerotic lesion progression and in determining plaque rupture. Apoptosis of inflammatory cells within an atherosclerotic lesion may result in their subsequent non-inflammatory, phagocytic removal from the plaque core, hence aiding the resolution of inflammatory processes occurring within the lesion, and potentially resulting in regression of the plaque itself. However, apoptosis in endothelial cells may worsen pre-existing endothelial dysfunction by removing any remaining functioning endothelial cells, whilst apoptosis in the VSMC population of the plaque cap may increase the likelihood of rupture in any given plaque.

1.4.3.1 Apoptosis of Inflammatory Cells in Atherosclerosis

Recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind plaque growth and development. However,

atherosclerotic plaques are dynamic and inflammatory cells are constantly turning over within the core. The identity of the cells responsible for clearing apoptotic macrophages from the plaque remains to be confirmed. It is currently unclear whether activated macrophages can themselves phagocytose populations of apoptotic inflammatory cells, or whether this is done by a subset of specialised phagocytes.

It is well established that apoptotic cells are present in atherosclerotic plaques. Apoptotic macrophages have been located by deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) staining in plaques from animal models of atherosclerosis and in plaques excised from human vessels (Han et al. 1995; Bjorkerud and Bjorkerud 1996; Haunstetter and Izumo 1998). The apoptotic macrophages tend to be clustered in areas of the plaque most vulnerable to rupture, in particular, the shoulder, or base, region, where the VSMC prevalence is also decreased (Kolodgie et al. 2000). It is currently unclear why these areas of the plaque are particularly susceptible to rupture, however, reduced VSMC localisation in these areas of vulnerable plaques may indicate that apoptosis of a variety of cell types, including VSMCs, plays a role in plaque instability (Kockx and Herman 1998).

Recently it has been suggested that macrophage MPO could have an important role in determining plaque vulnerability to rupture. MPO generates ROS including hypochlorous acid (HOCl) as part of its normal function during innate host defences (Klebanoff 1980; Hazen and Heinecke 1997). Sugiyama *et al* have described a strong co-localisation between macrophage MPO expression and HOCl-modified proteins at sites of lesion rupture in patients who suffer acute cardiac events

(Sugiyama et al. 2001). MPO-generated HOCl at relatively high physiological concentrations, but still within the range expected to be produced at areas of vascular inflammation (30–50 μM), has been shown to promote endothelial cell death by stimulating apoptotic pathways, including rapid caspase-3 activation and DNA fragmentation (Sugiyama et al. 2004). This observation suggests that, prior to undergoing apoptosis themselves, activated macrophages may induce apoptosis in neighbouring endothelial cells through MPO expression, ultimately resulting in plaque rupture.

1.4.3.2 Apoptosis of VSMCs In Atherosclerosis

Loss of VSMCs from the protective cap may be a major determinant of plaque susceptibility to rupture. Because healthy endothelial cells secrete factors that promote VSMC survival, a consequence of activated macrophage-induced endothelial cell death is an increase in VSMC death (Gordon et al. 1990). In addition to removing the protective presence of the endothelium, macrophages can also influence VSMC apoptosis directly. Activated human monocytes/macrophages have been found to be responsible for the death of human VSMC *in vitro* (Seshiah et al. 2002; Vasudevan et al. 2003). Seshiah *et al* have hypothesised that M-CSF, a haematopoietic growth factor supporting survival and differentiation of monocytes, is secreted from VSMCs resulting in macrophage activation, and ultimately triggering subsequent VSMC apoptosis (Seshiah et al. 2002). The exact mechanism of this process remains to be fully understood, but it is thought that macrophages prime VSMCs to respond to apoptotic stimuli, for example by triggering the

expression of death receptor ligands such as TNF-α (Mayr and Xu 2001). Direct induction of VSMC apoptosis in the *ApoE*$^{-/-}$ null murine model of atherosclerosis also induces both rupture and thrombosis of the plaque, independently of macrophage involvement. (von der Thusen et al. 2002).

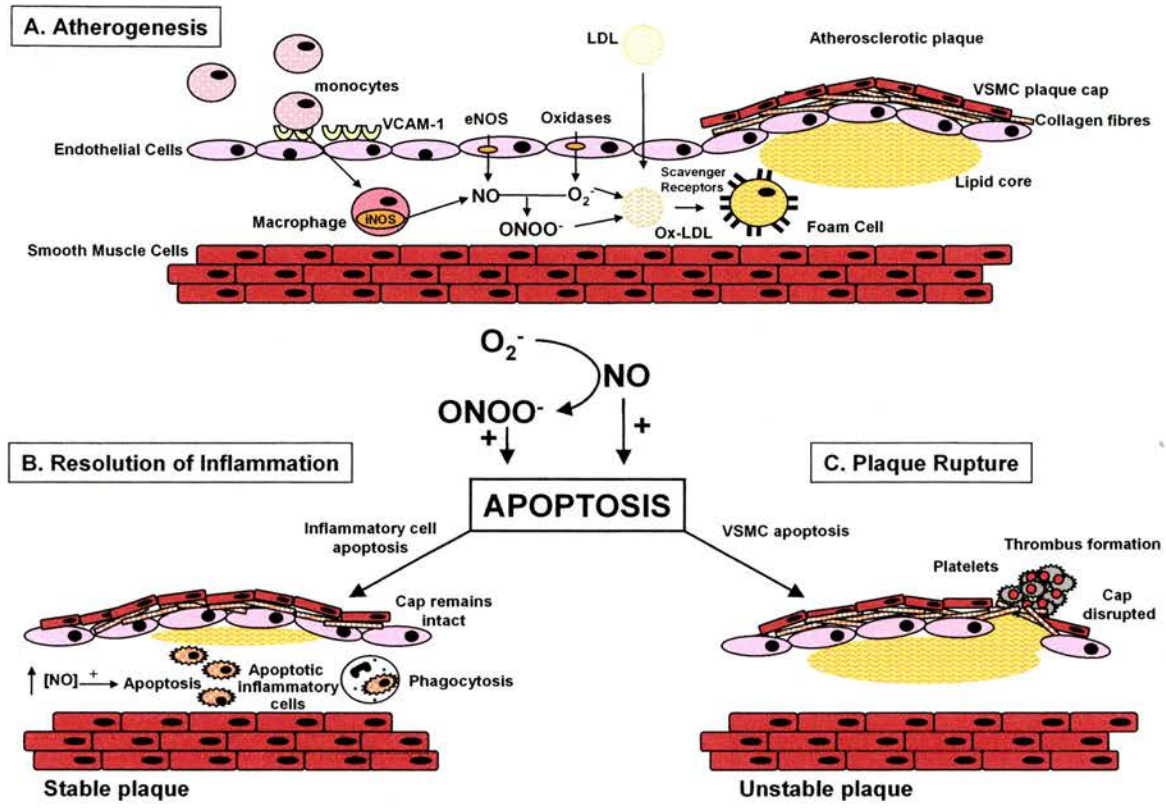

Figure 1.9 Processes Involved in Atherosclerosis

(A) During atherogenesis, circulating monocytes translocate to the sub-endothelial space where thay accumulate modified lipids to form an atherosclerotic plaque that becomes overlain with a fibrous cap of VSMCs and extracellular matrix fibres such as collagen

(B) Apoptosis can be induced in a variety of cell by NO. Apoptotic inflammatory cells are cleared by phagocytes, aiding resolution of the inflammatory response, stabilising the plaque and leading, ultimately, to plaque regression.

(C) Apoptosis of VSMCs may be detrimental, causing degradation of the lesion cap, particularly at the shoulder regions of the plaque, which can lead to plaque rupture, thrombus formation and ultimately to the acute clinical consequences of atherosclerosis, such as myocardial infarction and stroke.

1.5 NO and Apoptosis in the Resolution of Inflammation During Atherosclerosis

Given that apoptosis is now thought to be key to the successful resolution of the inflammatory response, pharmacological manipulation of apoptosis in a variety of cell types, particularly inflammatory cells, may represent a novel therapeutic strategy for the treatment of chronic inflammatory disorders (Ward et al. 1999; Gilroy et al. 2004). Inducing apoptosis in atherosclerosis could potentially delay disease progression, or even promote disease regression.

1.5.1 Therapeutic Potential of NO in Atherosclerosis

NO is a promising candidate for use in the treatment of atherosclerosis because its ability to induce apoptosis and aid inflammatory resolution has already been demonstrated in several animal models. In a mouse model of kidney inflammation, activated macrophages have been shown to induce apoptosis in neighbouring mesangial cells prior to ingestion by phagocytes (Duffield et al. 2000). The ability of the activated macrophages to induce apoptosis is greatly reduced in the presence of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), indicating that the macrophage-directed apoptosis of mesangial cells occurs via a NO-dependent mechanism (Duffield et al. 2001). Similarly, several studies have demonstrated that activated macrophages infiltrating murine tumours induce apoptosis via a NO-dependent pathway in both activated anti-tumour T cells and in the tumour cells themselves (Saio et al. 2001; Chattopadhyay et al. 2002). Thus, it

appears that macrophages have the capacity to induce apoptosis of nearby cells by the liberation of NO, or an NO-related species, to enhance the clearance of apoptotic cells.

Inducing apoptosis in inflammatory cells within an atherosclerotic lesion is an attractive prospect as it may represent a mechanism to resolve vascular inflammation, thereby stabilising the plaque and halting disease progression. Reducing the number of activated macrophages present in the plaque would have multiple consequences. Firstly, the physical presence of the plaque in the vessel would be decreased, reducing stenosis. Secondly, because activated macrophages induce apoptosis in neighbouring endothelial cells and VSMCs, reducing the number of activated macrophages may help to preserve endothelial function and maintain the integrity of the plaque cap, hence reducing the likelihood of plaque rupture. Considered in combination with the additional powerful anti-atherogenic characteristics of NO, including inhibition of platelet and inflammatory cell activation, these potentially beneficial pro-apoptotic properties of NO make this molecule an appealing prospect as a mechanism to regress atherosclerosis.

Support for the hypothesis that manipulation of apoptosis by NO could be used to reverse atherosclerosis is emerging from animal studies. For example, L-arginine (the substrate for NOS) or the NO donor sodium nitroprusside (SNP), administered to hypercholesterolemic rabbits increases the number of apoptotic macrophages present in intimal lesions by three fold (Wang et al. 1994; Wang et al. 1999). This increase in apoptosis was accompanied by a significant reduction in intimal thickening, demonstrating that manipulation of the NO synthase pathway, or

delivery of exogenous NO, may be a way to boost NO availability in order to stabilise, or even regress, the plaque via an apoptotic mechanism (Wang et al. 1994; Wang et al. 1999). However, such treatments are by no means selective for macrophages, and as already discussed, NO will induce apoptosis in endothelial cells and VSMCs. This could have several serious detrimental consequences for the plaque: firstly, additional loss of endothelial function would occur leading to further exacerbation of the disease process. Secondly, because VSMCs are essential for maintaining the integrity of the plaque cap, loss of cells in vulnerable regions of the lesion could destabilise the plaque and cause rupture. The potential benefit of regressing the plaque in this manner must also be offset against the cost of reducing the size of the macrophage population available for scavenging existing apoptotic macrophages, endothelial cells and VSMCs, which, if remaining *in situ*, will undergo secondary necrosis, thereby increasing the thrombogenicity of the plaque as a whole. In the study by Wang *et al* described above, apoptosis is quantified by the number of apoptotic nuclei per area of plaque, rather than as a percentage of the total macrophage population. Therefore, it is not possible to draw any conclusions as to whether the phagocytosis capacity of the plaque has been affected in this case.

Although human macrophages appear unable to generate the supraphysiological concentrations that murine cells produce (Albina 1995; Weinberg et al. 1995; Thomassen and Kavuru 2001; Schneemann and Schoedon 2002), human macrophages will respond to exogenous NO delivered by synthetic NO donor compounds, suggesting that NO could potentially be used to manipulate rates of apoptosis in human atherosclerosis. However, global, non-selective delivery of NO

concentrations sufficient to induce apoptosis are likely to cause systemic hypotension. Additionally, indiscriminate pro-apoptotic events could have serious adverse consequences for the plaque dynamic, resulting in a greater propensity to rupture, and thereby increasing the risk of myocardial infarction and stroke. It is therefore essential to target any therapeutic intervention to specific cell types within the plaque, and in particular, to appropriate cell types within those plaques vulnerable to rupture.

1.5.2 NO Donor Drugs

Organic nitrates have been used for over a century to treat angina (Parker and Parker 1998). However, the development of nitrate tolerance prevents the continuous use of this class of drugs (Csont and Ferdinandy 2005; Munzel et al. 2005). The mechanisms underlying development of tolerance and subsequent loss of therapeutic effect remain unclear. A number of hypotheses to explain these phenomena have been put forward but no consensus amongst investigators has been reached. Possibilities include impaired bioactivation (Sage et al. 2000), excessive compensatory levels of potent vasoconstrictors such as endothelin-1 (Munzel et al. 1995) and angiotensin II (Kamajian and Tilley 1975; Wada et al. 2002; Hirai et al. 2003), increased O_2^- production (Munzel and Harrison 1997; Fayers et al. 2003), and a desensitisation of sGC (Artz et al. 2002). Similarly SNP has been used to treat acute hypertension but its therapeutic potential is limited by a host of difficulties in the clinical setting, including the necessity for intravenous administration, difficulties with dose titration, and the accumulation of toxic cyanide metabolites (Friederich

and Butterworth 1995; Megson 2000). Such problems with traditional NO donor drugs, together with the observation that endogenous NO production is often decreased in cardiovascular disease, has led to the development of several categories of novel synthetic NO donor compounds with the aim of delivering exogenous NO, stimulating sGC, or elevating cGMP levels within cells. The development of such compounds represents not only the prospect of potential therapies for conditions associated with decreased endogenous NO production, but also provide invaluable experimental tools in the quest to elucidate the mechanisms underlying such conditions.

Cell specificity will be critical to the success of any NO-based therapy in atherosclerosis. Existing NO donor drugs are not selective for particular cell types; commonly used organic nitrates, such as glyceryl trinitrate (GTN), tend to have an unfavourable selectivity profile when considered in the context of atherosclerosis (veins > arteries > platelets; MacAllister et al. 1995; Kojda et al. 1998; Barba et al. 1999). In order to use NO donor drugs as effectors of apoptosis in atherosclerosis, compounds that are able to discriminately act on, for example macrophages, but not endothelial cells or VSMCs, will have to be developed in the future. One possibility may be to target macrophage iNOS. However, whilst human macrophages do express iNOS mRNA and protein (Weinberg et al. 1995), so too do VSMCs and endothelial cells present in atherosclerotic plaques (Wilcox et al. 1997; Yan and Hansson 1998).

In addition to selectivity, another major consideration when contemplating the use of NO donor drugs for the treatment of atherosclerosis is that of delivery. A

global, non-selective release of NO throughout the circulation may have various anti-atherosclerotic actions, but dosing will be limited by concurrent vasodilatation, resulting in systemic hypotension. The challenge, therefore, is to generate high local concentrations of NO in the immediate vicinity of a plaque. Possible means of achieving this include the use of NO donor drugs that are selective for areas of endothelial damage, such as RS-N=O (see section 1.5.2.2), or use of NO-eluting stents to prevent restenosis following angioplasty. Angioplasty is a common clinical intervention to improve blood flow through stenosed (or occluded) arteries, however, restenosis of the vessel currently limits the success of the procedure (Liu et al. 1989; Preisack and Karsch 1993). Given that NO inhibits VMSC proliferation (Newby et al. 1992; Mooradian et al. 1995; Jeremy et al. 1999), NO-eluting stents may limit restenosis by inhibiting intimal hyperplasia, as well as treating the underlying condition by providing all the anti-atherogenic properties of NO *in situ.* There has been some success to date in humans with the use of sirolimus (rapamycin)-eluting stents in preventing the neointimal proliferation which leads to re-stenosis following angioplasty, although the long-term effectiveness (>1 year) remains to be established (Sousa et al. 2001). Nevertheless, this demonstrates that use of drug-eluting stents may be a practical application in this specific clinical setting.

A further potential method of delivering high local concentrations directly to the interior of the plaque may be to exploit the lipid environment of the core by developing novel lipophilic NO donor drugs designed to induce apoptosis in the lipid laden macrophages within the core.

1.5.2.1 Diazeniumdioloates

The diazeniumdiolate compounds, or 'NONOates', are complexes of NO with nucleophilic adducts that are considered to be 'pure' NO donors. These compounds release up to two molecules of NO per molecule of compound spontaneously in solution and, unlike other NO donor drugs such as SNP, do not require tissue thiol or enzymatic activation (Diodati et al. 1993; Megson 2000; Megson and Webb 2002). Biological efficacy can be accurately predicted from their *in vivo* rate of decomposition which follows first order kinetics, and is determined by the nucleophilic adduct, temperature, and pH (Morley and Keefer 1993; Megson 2000). Varying the nucleophilic adduct has produced a diversity of compounds with differing rates of decompositions, ranging from locally active drugs which release their NO within minutes, for example, 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO; t½ = 2 min; figure 1.10 A), to longer acting compounds which generate NO over a period of several hours, for example (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO; t½= 20 h; figure 1.10 B).

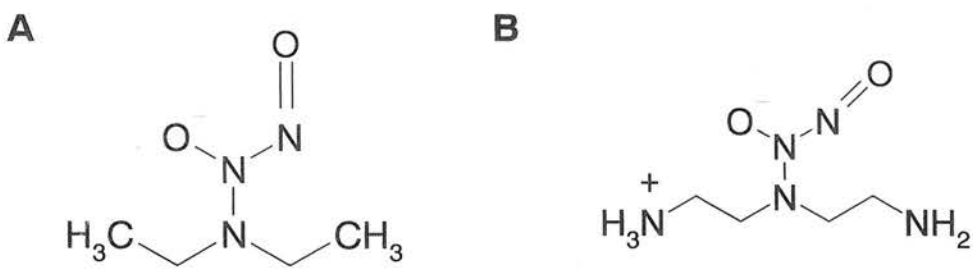

Figure 1.10 The Chemical Structures of DEA/NO (A) and DETA/NO (B).

1.5.2.2 S-Nitrosothiols

S-nitrosothiols, general formula RS-N=O, are formed endogenously and can be synthesised by nitrosation (transfer of NO^+) of reduced thiols (Butler and Rhodes 1997); examples include endogenous GSNO (figure 1.11 A) and the synthetic compounds S-nitroso-N-acetylpenicillamine (SNAP) and S-nitroso-N-valeryl-penicilliamine (SNVP; figure 1.11 B). The biological half-life of S-nitrosothiols is unpredictable in solution and dependent on the R group of the compound (Mathews and Kerr 1993). The exact mechanism of decomposition and subsequent NO release from S-nitrosothiols, both endogenous and synthetic, is not fully known. However, decomposition is greatly accelerated in the presence of copper I (Cu^+) ions (Gordge et al. 1995; Dicks and Williams 1996; Gorren et al. 1996; Singh et al. 1996). Interestingly, the enzyme sGC contains one mole of copper per mole of holoenzyme dimer (Ignarro 1992), which may act as a catalyst to trigger NO release from endogenous S-nitrosothiols. *In vivo*, S-nitrosothiols are thought to act via transfer of NO^+ to reduced tissue thiols without the release of free NO radical (Broillet 1999). A recent study has shown that the cell surface protein, disulfide isomerase, may act as a chaperone molecule to transfer S-nitrosothiol-derived NO from extra-cellular S-nitrosothiols into the cell cytosol (Ramachandran et al. 2001).

S-Nitrosothiols are generally accepted to be platelet-selective NO donor drugs (de Belder et al. 1994) and some have been shown *in vitro* to have vasodilator actions which are selective for areas of experimentally denuded endothelium (Megson et al. 1997; Megson et al. 1999; Sogo et al. 2000b). Furthermore, in a rabbit balloon angioplasty model of vascular injury, SNVP, in contrast to the

traditional organic nitrate, GTN, reduced the adhesion or radiolabelled platelets in areas of endothelial damage without significantly affecting systemic blood pressure (Miller et al. 2003). This indicates that S-nitrosothiols may have advantages over other NO donor drugs for the treatment of atherosclerosis, as they are able to discriminate areas of endothelial damage, which is an important precursor to plaque development.

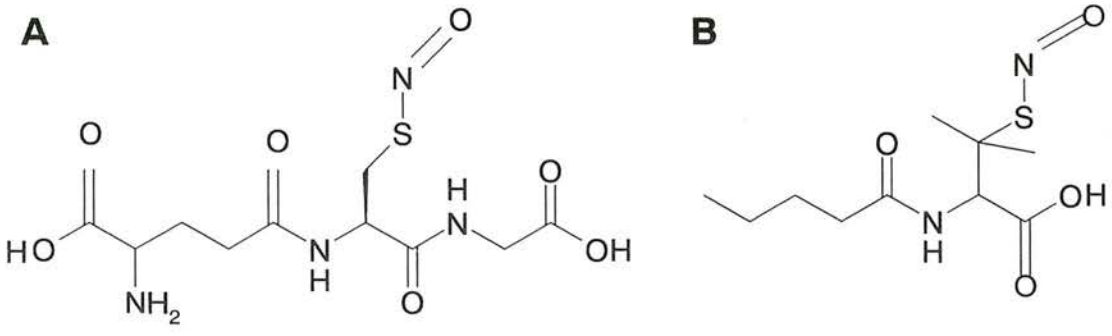

Figure 1.11 The Chemical Structures of GSNO (A) and SNVP (B)

1.5.2.3 Mesoionic Oxatriazole Derivatives

The mesoionic oxatriazole derivatives and sydnonimines are not strictly NO donors but are generators of the NO-related species, $ONOO^-$. Molsidomine is the precursor of 3-morpholinosyndnonimine (SIN-1). Although originally considered to be a NO donor, SIN-1 is now widely accepted to be a $ONOO^-$ generator on account of concomitant generation of equivalent concentrations of NO and O_2^- which rapidly combine to form $ONOO^-$ (Feelisch et al. 1989). Mesoionic 3-aryl, 5-imino-oxatriazole derivatives, for example GEA-3162 (figure 1.12), represent a novel class of NO donors which are structurally very similar to the sydnonimines (Megson 2000; Megson and Webb 2002). Until recently, GEA-3162 was regarded as a pure NO

donor (Kankaanranta et al. 1996; Holm et al. 1998), but there is now clear evidence that, in common with the structurally similar SIN-1, GEA-3162 is a $ONOO^-$ generator rather than an NO donor (Taylor et al. 2004).

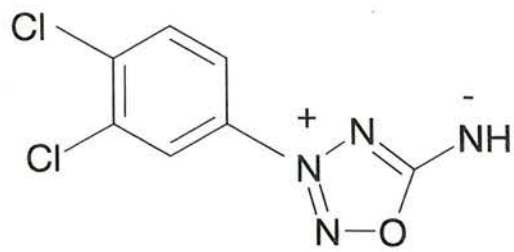

Figure 1.12 The Chemical Structure of GEA-3162

1.5.2.4 NO-independent Stimulators of sGC

Due to the uncertainty regarding the exact NO-related species responsible for many of the effects of NO, coupled with the drawbacks of existing NO-based therapies, major efforts have been made to develop NO-independent stimulators of sGC. 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl-indazol (YC-1) was the first such compound developed which displayed anti-platelet properties via elevation of intracellular cGMP (Ko et al. 1994; Wu et al. 1995). More recently, following the discovery of an NO-independent regulatory site on sGC, BAY 41-2272 (figure 1.13) was developed (Stasch et al. 2001). BAY 41-2272 is a pyrazolopyridine, which has been reported to potently stimulate sGC through the NO-independent, haem-dependent regulatory site on the α_1-subunit of the enzyme in order to produce both anti-platelet actions and the ability to decrease blood pressure in a rat model of hypertension (Stasch et al. 2001). However, more recently the physiological effects

of both YC-1 and BAY 41-2272 have been attributed to the synergistic effects of inhibition of the cGMP-inactivator, phosphodiesterase 5 (PDE V), coupled with sensitisation of sGC toward endogenous NO (Friebe et al. 1998; Mullershausen et al. 2004).

Whilst compounds such as these may overcome some of the difficulties associated with more traditional NO donor drugs, it should be noted that NO is able to exploit pathways independently of cGMP signalling and that these additional pathways would be unavailable to NO-independent stimulators of sGC.

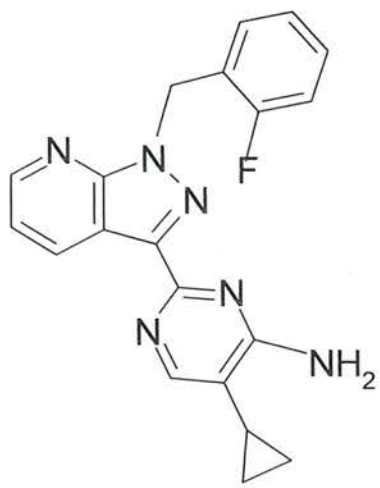

Figure 1.13 The Chemical Structure of BAY 41-2272

1.6 Summary

Atherosclerosis has a highly complex pathogenesis and is a condition that evolves over many years. Initiated by the retention of pathogenic lipids and consequent insult to the endothelial cell layer of blood vessels, the disease progresses via activation of a chronic inflammatory response involving the recruitment and

activation of inflammatory cells, such as macrophages, which go on to internalise ox-LDL and accumulate in the sub-endothelial space of the vessel to form a fatty streak. The fatty streak progresses to the hallmark atherosclerotic plaque through the recruitment and activation of further inflammatory cells, which become encapsulated by a cap of VSMCs and fibrous matrix. The VSMC cap acts as barrier between the highly thrombogenic core of the plaque and the circulation. The major determinant of outcome in atherosclerosis is now considered to be plaque rupture. The mechanisms involved in plaque vulnerability to rupture are not yet fully understood but are thought to include inflammatory processes occurring within the plaque, and the thickness and ability of the cap overlaying the lesion to withstand mechanical erosion.

Apoptosis is a fundamental biological process governing cell survival and is thought to be critical to the successful resolution of the inflammatory response. Apoptotic cells are removed from the site of inflammation by non-inflammatory phagocytosis. Manipulation of apoptosis in a variety of cell types may aid the resolution of the inflammatory response occurring in atherosclerosis, hence halting the disease process or even aiding plaque regression.

The endogenous free radical signalling molecule, NO, has a complex chemistry and its effects may be elicited by a variety of intermediary NO-related species that have biological actions of their own. The anti-atherogenic properties of NO include regulation of vascular tone and inhibition of platelet and inflammatory cell activation. In addition to this, NO can be both pro- and anti-apoptotic depending on the NO-related species and cell type in question. NO-induced manipulation of

apoptosis in atherosclerosis, particularly in macrophages, may be beneficial in terms of reducing plaque size, but this benefit must be offset against the potential risk of indiscriminate apoptotic events de-stabilising the plaque and causing rupture.

1.7 Thesis Aims

The aims of this thesis were to characterise the precise NO and NO-related species liberated by various synthetic putative NO donor compounds and to examine their role in various processes occurring in cell types relevant to atherosclerosis at concentrations which might be predicted to be physiologically relevant.

The central hypotheses addressed will be:

1. Three different classes of putative NO donor compounds – diazeniumdiolates, RS-N=O, and mesoionic oxatriazole derivatives – will decompose in solution to generate different NO and NO-related species.
2. Different NO-related species will exert disparate effects on the proliferation of VSMCs.
3. Variations in the actions NO-related specis can be exploited to differentially induce apoptosis in different cell types, namely VSMC and human macrophages.
4. Elevating cGMP in VSMC and human macrophages will result in cellular protection against subsequent apoptotic cell death.

Chapter Two

Materials and Methods

2. Methods

2.1 Detection of NO

2.1.1 Electrochemical Detection of NO

NO released in solution from the NO donors DEA/NO, DETA/NO, SNVP, GSNO, and GEA-3162, was measured using an isolated NO electrode (Iso-NO II, World Precision Instruments, Stevenage, U.K), calibrated daily with DEA/NO (0.1 - 1.6 μM) in phosphate buffer (pH 4; 2ml; figure 2.1.A). DEA/NO has a rapid and predictable rate of decomposition at pH 4 (Davies et al. 2001). Therefore, the change in peak NO signal can be used to generate a calibration curve assuming a 2:1 stoichiometry of NO release from DEA/NO under the calibration conditions (Davies et al. 2001) and fitting the curve by linear regression (figure 2.1.B). In order to measure higher NO concentrations, the electrode sensitivity was decreased and re-calibration was performed with DEA/NO (1 – 16 μM; pH 4 phosphate buffer; 2 ml). In this situation, a slightly curved calibration graph was generated, therefore, the slope was fitted by second order polynomial nonlinear regression (figure 2.1.C and 2.1.D). Data were captured via a MacLab 4/e analogue-digital converter and displayed through Chart™ *v* 3.6/s software (AD Instruments, Sussex, U.K).

Prior to the addition of NO donor compounds, aliquots (2 ml) of tissue culture media were pre-warmed (37°C) and stirred (600 rpm) until a stable electrode baseline had been achieved. NO released from each NO donor compound was measured in the presence or absence of Cu/Zn SOD (50 – 500 $U.ml^{-1}$). At the end of each recording, the recognised NO scavenger, haemoglobin (Hb; 5 μM; Hille et al. 1977), was introduced into the electrode chamber to quench the signal and verify the

presence of NO. The concentration of NO generated was calculated from the slope of the calibration graph, or by solving the quadratic equation for the calibration curve produced when the electrode sensitivity was reduced.

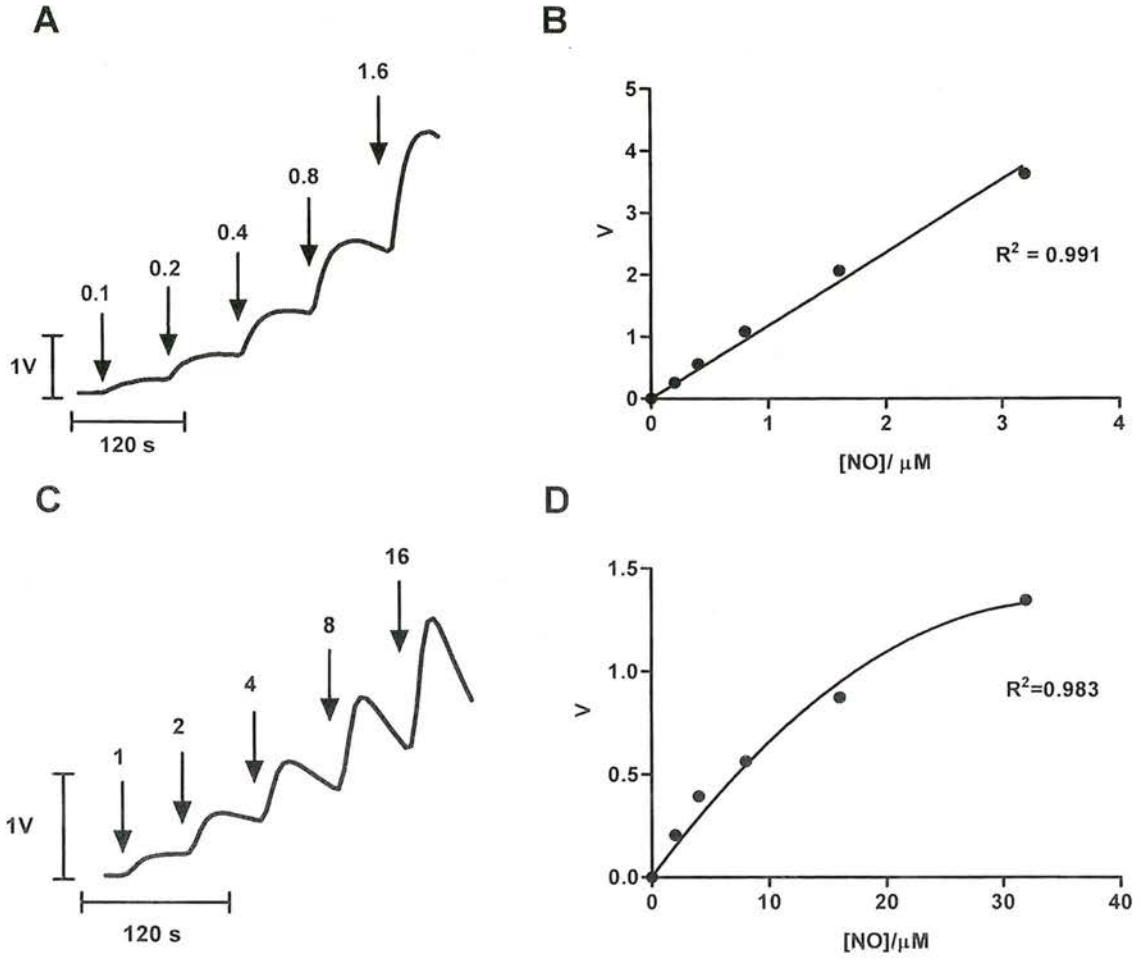

Figure 2.1 NO Electrode Calibration.

Example single calibration trace (A) and graph (B) with electrode at high sensitivity setting. In order the measure higher concentrations of NO, the sensitivity was reduced and the electrode re-calibrated (C), generating a curved calibration graph (D). DEA/NO (μM) additions are indicated by arrows.

2.1.2 Detection of NO_2^-/NO_3^- by Griess Test

The total nitrite/nitrate (NO_2^-/NO_3^-; NOx) concentration released by SNVP, GSNO, and GEA-3162 (all 10 – 300 μM) in tissue culture media in presence of bovine aortic smooth muscle cells (BAoSMC) or human monocyte-derived macrophages, and that released by human monocyte-derived macrophages stimulated by lipopolysaccharide (LPS; 1 $\mu g.ml^{-1}$), was measured by Griess Test (R&D Systems, Abington, U.K). It was not possible to measure NOx generation from DEA/NO and DETA/NO by Griess reaction because reagents are acidified during the protocol and NO release from diazeniumdiolate compounds is pH dependent (Davies et al. 2001).

The Griess reaction allows colourimetric detection of NO_2^- as an azo dye product that absorbs light at 540 nm. NO_2^- in the sample reacts with sulfanilic acid to produce the diazonium ion, which is then coupled to N-(naphthyl) ethylenediamine to form the chromophoric azo derivative. Measurement of total NOx is achieved by enzymatic conversion of NO_3^- to NO_2^- by nitrate reductase prior to addition of Greiss reagents.

Aliquots (200 μl) of tissue culture media were aspirated at 1h, 6h, and 24h time points from tissue culture plates containing BAoSMC or human monocyte-derived macrophages (see sections 2.2.3 and 2.2.2.1) in the presence or absence of NO donor compounds, or human monocyte-derived macrophages stimulated with LPS (1 $\mu g.ml^{-1}$). Aliquots were frozen immediately on dry ice and stored (-70°C) for approximately 1 week prior to performing the assay. For the NO_2^- assay, absorbance (540nm) was measured in 96-well, flat-bottomed microtiter plates following incubation (10 min; room temperature; RT) with Griess Reagent I (sulfanilamide in 2

M hydrochloric acid; HCl) and Greiss Reagent II (*N*-(1-naphthyl) ethylenediamine 2 M in HCl). For the NOx assay, samples were incubated (37°C; 30 min) in the presence of the enzyme nitrate reductase and its substrate β-nicotinamide adenine dinucleotide (NADH) in 96-well, flat-bottomed mircotiter plates. Following this incubation period, samples were assayed as for NO_2^- as above. The absorbance (540 nm) of the relevant tissue culture media was measured and this value was used as a blank that was subtracted from all readings. The concentration of total NOx was calculated from the slope of standard curves generated daily by serial dilution of known concentrations of sodium nitrite (3.12 – 200 μM) and sodium nitrate (3.12 – 100 μM; figure 2.2). All standards and samples were assayed in duplicate. Optical densities (540 nm) were measured by a Multiskan Ascent plate reader and data captured using Ascent™ v 2.6 software (Thermo Labsystems, South Trentham, U.K).

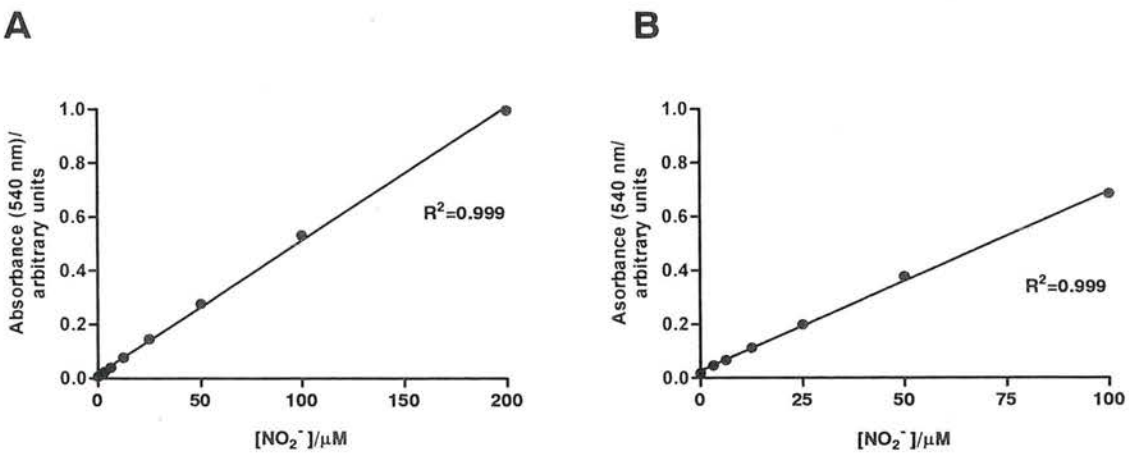

Figure 2.2 Standard Curves Generated for the Calculation of NO_2^-/NO_3^- Concentration

Example standard curves generated by the absorbance (540 nm) of sodium nitrite (0 – 200 μM; A) and sodium nitrate (0 – 100 μM; B) following conversion to NO_2^- by nitrate reductase.

2.1.3 Detection of NO_2^-/NO_3^- by High Performance Liquid Chromatography

Concentrations of NO_2^-/NO_3^- below the limit of detection of the Griess test (<3 μM) were measured by fluorescence high-performance liquid chromatography (HPLC), which allows detection of NO_2^- in the nM range . The fluorometric assay is based on the reaction of NO_2^- with 2,3-diaminonaphthalene (DAN) under acidic conditions to yield the fluorescent product, 2,3-naphthotriazole (NAT), which is stable under alkaline conditions (Misko et al. 1993; Li et al. 2000; Woitzik et al. 2001; Gharavi and El-Kadi 2003).

Aliquots (500 μl) of tissue culture media were aspirated from wells containing LPS- stimulated (1 $\mu g.ml^{-1}$; 24 h) human monocyte-derived macrophages. Aliquots were frozen immediately on dry ice and stored (-70°C) for approximately one week prior to performing the assay.

Prior to detection of NO_2^-, samples were filtered through pre-washed ultrafilter centrifuge tubes (12 kD pore size) in order to remove any protein. NO_2^- was detected by incubation (10 min; RT; light proof conditions) of each sample with DAN under acidic conditions (0.125 $\mu g.ml^{-1}$ in 0.7 M HCl). Following this period, the pH of each sample was raised by addition of sodium hydroxide (NaOH; 1.7 M), and the sample stored (5°C) prior to analysis by HPLC. In order to calculate the concentration of NO_2^-, a calibration curve was generated daily from the fluorescence peak area of serial dilutions of known concentrations of sodium nitrite (0.1 – 1.0 μM; figure 2.3). Due to difficulties with the solubility of DAN, a curved calibration graph was generated, therefore the line was fitted by second order polynomial nonlinear

regression and the concentration of NO_2^- generated in subsequent experiments calculated by solving the quadratic equation of the curve.

NO_3^- would normally be measured following chemical conversion to NO_2^- by copper-coated cadmium prior to analysis by HPLC. However, this reaction did not occur in the tissue culture media in which the human monocyte-derived macrophages were cultured. To overcome this problem, an attempt was made to combine enzymatic conversion (nitrate reductase) of NO_3^-, with fluorescence HPLC detection of NO_2^-. Each sample was incubated (37°C; 30 min) in presence of nitrate reductase (1 $U.ml^{-1}$) and its substrate nicotinamide adenine dinucleotide phosphate (NADPH; 80 μM). Serial dilutions of known concentrations of sodium nitrate (1-10 μM) were also treated in this manner in order to generate a standard curve. Following this incubation period, samples were assayed for NO_2^- as described above. However, the detection of NO_2^- following this system of converting NO_3^- to NO_2^- was unsuccessful. This is likely due to interference by the nitrate reductase co-factors, $NADP^+$/NADPH, with NAT, resulting in a quenching of the fluorescence signal (Woitzik et al. 2001). This interference could potentially be avoided during future attempts to convert NO_3^- to NO_2^- enzymatically by addition of D-glucose-6-phosphate monosodium salt and glucose-6-phosphate dehydrogenase, which act to aid the recycling $NADP^+$ to NADPH. This system has been successfully employed by others to prevent the interference, and subsequent reduction in fluorescence formation by NAT, caused by $NADP^+$ (Woitzik et al. 2001). However, as this system was not used here, it was not possible to measure NO_3^- by this HPLC method.

The HPLC system consisted of a multi wavelength fluorescence detector (2475 model, Waters, Massachusetts, USA; λ excitation = 365 nm, λ emission = 425

nm). The mobile phase, 65% borate buffer: 40% acetonitrile, was pumped through the system at a flow rate of 1 $ml.min^{-1}$ using a Waters 2695 Separations Module with integrated autosampler (Waters, Massachusetts, USA). An Xterra column and a HPLC guard-column insert packed with C18 (Waters, Massachusetts, USA) were used for the assay. Data were captured and recorded using Empower™ 2 software (Waters, Massachusetts, USA). All solutions were of HPLC grade quality.

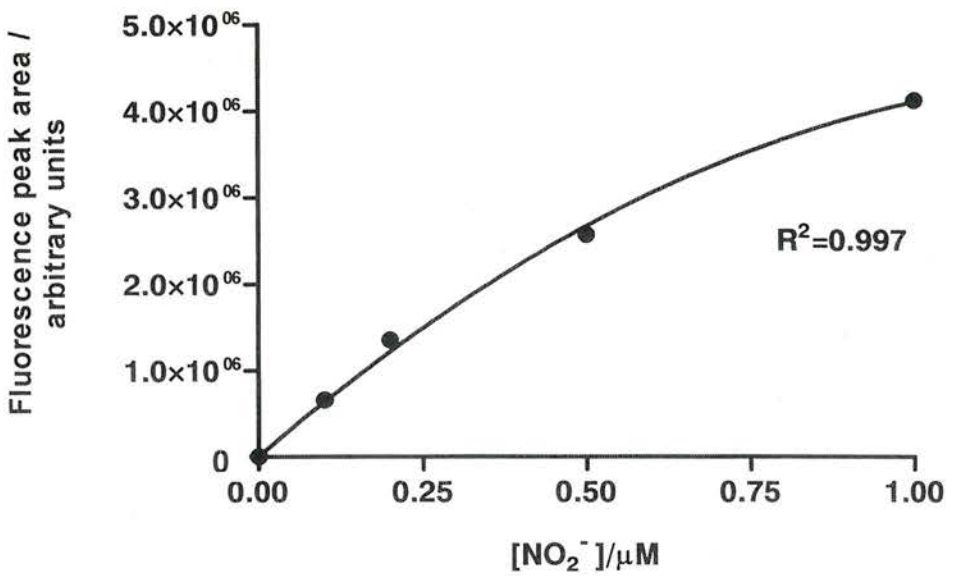

Figure 2.3 Standard curve generated for the calculation of NO_2^- concentration

Example standard curve generated from the fluorescence peak area produced by sodium nitrite (0.1 – 1 μM)

2.1.4 Electron Paramagnetic Resonance

The concentration of oxidising free radical species generated by DEA/NO, DETA/NO, SNVP, GSNO, and GEA-3162 in tissue culture media was assessed by electron paramagnetic resonance (EPR) spectrometry. This technique uses a chemical spin-trap, which reacts with free radical species to form a stable radical adduct. EPR spectrometry then measures the absorption of electromagnetic (microwave) radiation by the stable radical adduct when it is placed in a strong magnetic field. In these studies, the chèmical spin-trap used was Tempone-H hydrochloride (1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine . HCl), which reacts with oxidising species to form the stable radical adduct 4-oxo-tempo. Tempone-H has previously been demonstrated to be an effective spin-trap for oxidising radical species such as $O_2^{\cdot-}$, and has also successfully been used for the detection of $ONOO^-$ in biological systems (Dikalov et al. 1997a; Dikalov et al. 1997b).

Each NO donor compound (10 – 300 μM) was incubated (37°C; 30 min) in tissue culture media in the absence or presence of Cu/Zn SOD (500 $U.ml^{-1}$) together with the recognised spin trap for oxidising radical species, Tempone-H (1 mM; prepared in 10 mM ethylene diamine tetraacetic acid (EDTA) solution to minimise metal ion-induced auto-oxidation of the spin trap). Aliquots (50 μl) were removed from the incubation vessels at timed intervals (1, 10, and 30 min) and the intensity of signal corresponding to formation of the radical adduct 4-oxo-tempo (triplet centred around 3360 G) recorded by EPR spectrometry. EPR signals were measured using a Miniscope MS100 X-band spectrometer (Magnettech, Berlin, Germany) with the following parameter settings: field sweep 51.2 G, microwave frequency 9.5 GHz, microwave power 20 mW, modulation amplitude 1500 mG. Data were captured,

recorded, and manipulated via Multiplot™ 2.24 and Analysis™ 2.02 software (Magnettech, Berlin, Germany). Control experiments in the absence of NO donor, or radical-generating compounds were conducted in all cases and small signals were detected due to the auto-oxidation of Tempone-H to 4-Oxo-Tempone. This phenomenon was minimised by preparation of the spin trap, Tempone-H, in EDTA solution, which protected the spin trap against metal ion-induced oxidation. Control signals generated by auto-oxidation of Tempone-H were subtracted from the corresponding experimental signals.

2.2 Cell Culture

2.2.1 THP-1 Macrophage Cell Culture

Cells of the human monocyte/macrophage cell line, THP-1, were cultured (37°C; 95% O_2: 5% CO_2; 75 cm^2 tissue culture flasks) from cyropreserved stocks in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with penicillin (100 $U.ml^{-1}$), streptomycin (100 $U.ml^{-1}$), L-glutamine (200 mM) and heat inactivated foetal calf serum (FCS; 10% v:v). Prior to experimental assay, cell suspensions of this non-adherent cell line were aspirated from the flasks and a cell pellet obtained by centrifugation (220 *g*; 5 min). The pellet was then re-suspended (1ml; RPMI 1640 medium plus supplements) and viable cell yield obtained by trypan blue exclusion and use of a bright line haemocytometer. An aliquot (10 µl) of the cell suspension was mixed with trypan blue (190 µl: 1:20 dilution) and an aliquot (10 µl) of the resulting solution placed under the haemocytometer cover slip. Cells excluding trypan blue in the outer four corners of the haemocytometer were counted by light

microscopy (x10 magnification; figure 2.4). The number of cells present in 1 ml was then calculated according to the equation shown in figure 2.4.

Cells were then re-suspended at a density of 0.75 x 10^6 cells/ml and seeded (150 µl/well; 1.125 x 10^5 cells/well) in 96-well, round-bottomed, Flexiwell plates (BD Falcon) in the presence and absence of each NO donor compound or in the presence of the maximum concentration of the drug vehicle, dimethyl sulphoxide (DMSO; 1%).

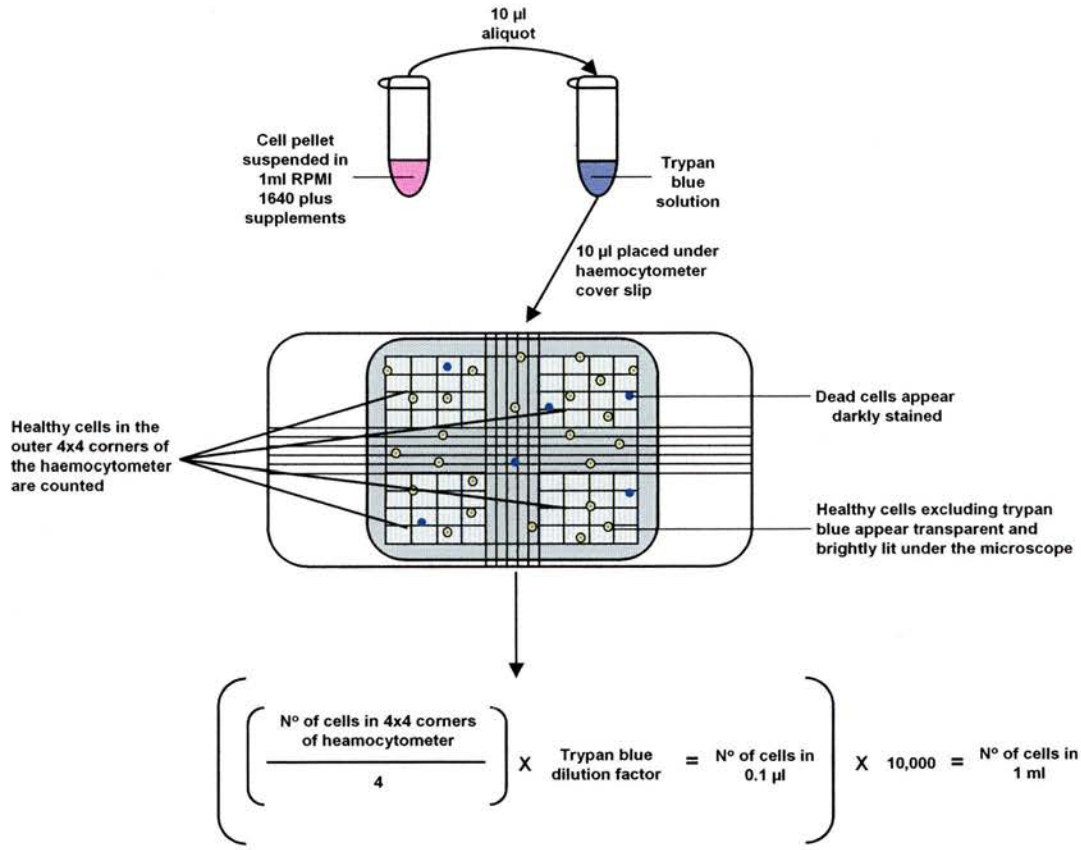

Figure 2.4 Assessment of Viable Cell Yield by Trypan Blue Exclusion

Cells excluding trypan blue are counted by light microscope (x10 magnification) and the cell yield calculated according to the equation shown.

2.2.2 Isolation of Human Mononuclear Cells

Human mononuclear cells were isolated from peripheral blood of healthy volunteers aged between 21 and 64. Venous blood (40 ml) was drawn from the antecubital fossa through a 19 gauge needle into 50 ml polypropylene tubes (BD Falcon) containing sterile sodium citrate (0.38% final concentration). Platelet rich plasma (PRP) was separated from whole blood by centrifugation (350 *g*; 20 min) and aspirated from the leucocyte/erythrocyte-rich layer (figure 2.5). Autologous serum was prepared in sterile glass tubes by incubating PRP (37°C; ~30 min) in the presence of calcium chloride ($CaCl_2$; 20 μM), which caused the platelets to aggregate. At the end of this incubation period the autologous serum was aspirated from the plug of aggregated platelets and stored (4°C) in polypropylene tubes.

Sedimentation of erythrocytes from the remaining cellular fraction was carried out by addition of 2.5 ml of pre-warmed (37°C), Dextran T500 (6%) per 10 ml haematocrit. Following addition of the appropriate volume of Dextran, the total volume of each tube was adjusted to 50 ml with pre-warmed (37°C) saline solution (0.9%). Erythrocytes were allowed to sediment for no longer than 30 minutes (RT), following which the leucocyte-rich upper layer was gently aspirated into 50 ml polyproylene tubes and the volume adjusted to 50 ml with pre-warmed (37°C) saline solution (0.9%). The leucocyte pellet was then obtained by centrifugation (350 *g*; 6 min).

Isotonic Percoll solution was prepared as a 9:1 (v:v) ratio of Percoll: 10x phosphate buffered saline (PBS) without calcium (Ca^{2+}) or magnesium (Mg^{2+}). The resulting solution was then used to prepare further solutions of 55%, 68%, and 81% isotonic Percoll in 1x Ca^{2+}/Mg^{2+}-free PBS. Discontinuous gradients were prepared

by layering 3 ml of 68% Percoll solution onto 3 ml of 81% Percoll solution in 15 ml polypropylene tubes. The leucocyte pellet was then re-suspended in 3 ml of the 55% Percoll solution and layered on top of the 68% solution to form the upper layer of the gradient. Sub-populations of leucocytes were separated by centrifugation of the gradients (720 *g*; 20 min; deceleration 0). Mononuclear cells were harvested at the 55:68% interface and washed twice in 1x PBS without Ca^{2+}/Mg^{2+} ions by centrifugation (220 *g*; 6 min). Granulocytes were harvested at the 68:81% interface.

Following the final wash, the isolated mononuclear cells were re-suspended (1x Ca^{2+}/Mg^{2+}-free PBS; 50 ml) and cell yield assessed by use of a bright line haemocytometer. An aliquot (10 µl) of the cell suspension was placed under the haemocytometer cover slip, the number of mononuclear cells in the central 25 squares counted by light microscopy (x10 magnification) and the cell yield calculated according to the equation shown in diagram 2.6. When the total yield had been obtained, the cells were centrifuged (220 *g*; 6 min), the PBS supernatant discarded and the cells re-suspended at a density of 4 x 10^6 cells/ml in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with penicillin (100 $U.ml^{-1}$) and streptomycin (100 $U.ml^{-1}$). The typical mononuclear yield was approximately 100 $x10^6$ cells from 160 ml whole blood, although this varied considerably between donors.

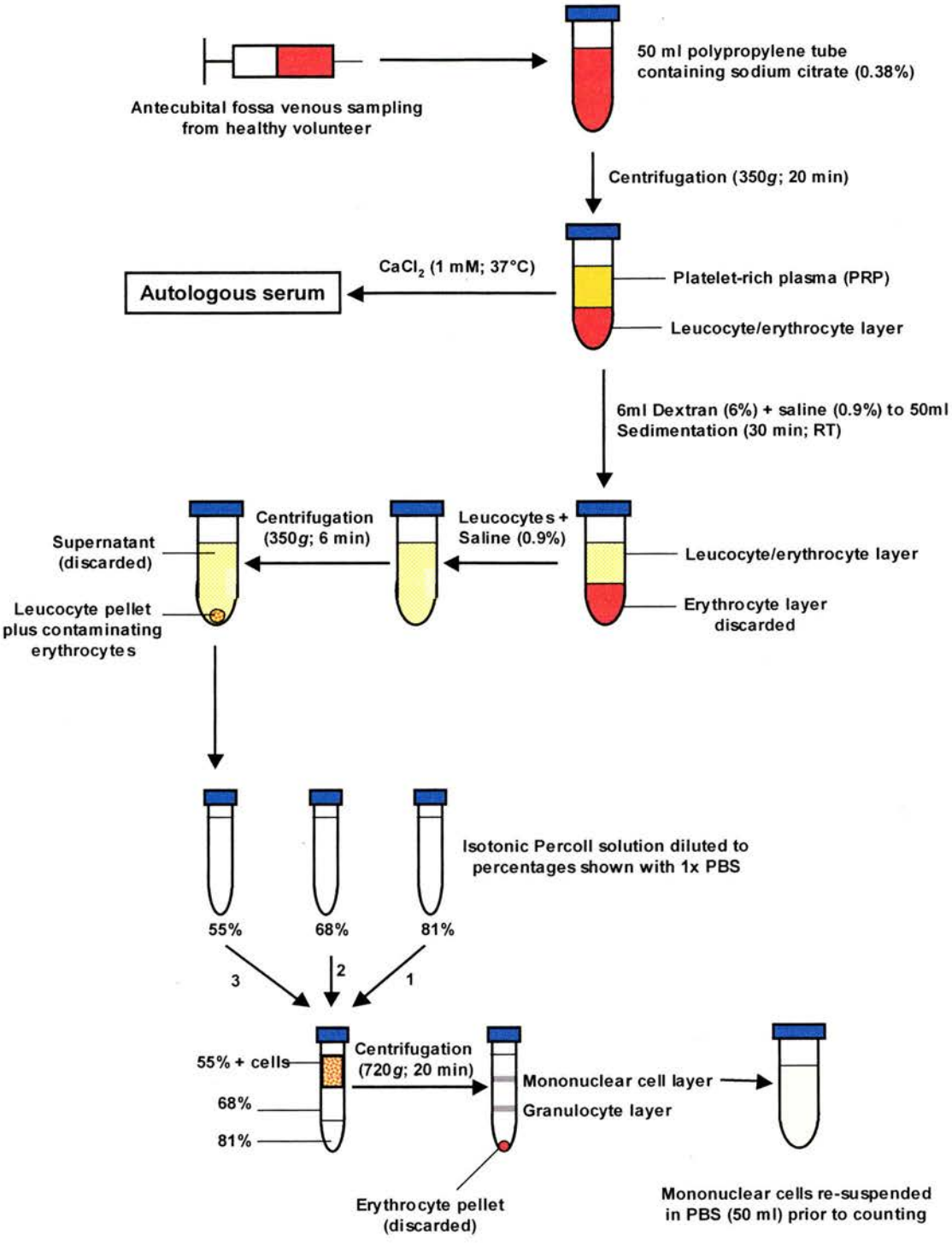

Figure 2.5 Isolation of Human Mononuclear Cells.

Whole blood was separated into platelet-rich plasma (PRP) and erythrocytes by centrifugation. Leucocytes were separated from erythrocytes by Dextran sedimentation. Finally, leucocyte sub-populations were separated by centrifugation through discontinuous Percoll gradients and the mononuclear cells re-suspended in PBS prior to assessment of cell yield.

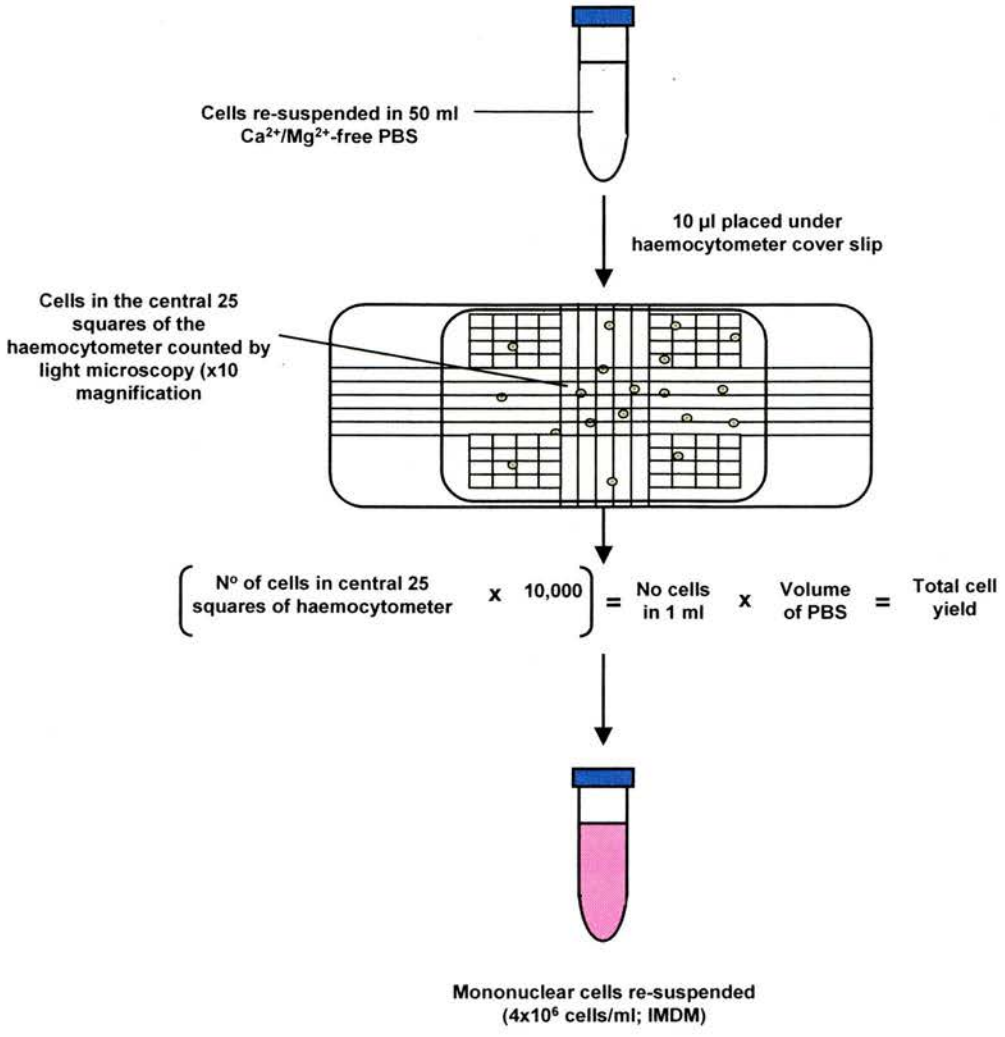

Figure 2.6 Assessment of Total Mononuclear Cell Yield

Cells present in the central 5 squares of the haemocytometer were counted by light microscopy (x10 magnification), and the cell yield calculated by the equation shown prior to re-suspension of the cells in IMDM ($4x10^6$ cells/ml).

2.2.2.1 Human Mononuclear Cell Culture

Re-suspended cells (4 x 10^6 cells/ml in IMDM) were seeded in 48-well tissue culture plates at a density of 2 x 10^6 cells/well (2.1 x 10^5 cell/mm^2) and incubated (37°C; 95% O_2: 5% CO_2) for 1 hour. During this initial incubation period the monocyte cell population adheres to the plate. After 1 hour the tissue culture medium was aspirated and the adherent cells washed twice with Hank's Balanced Salt Solution (HBSS; containing Ca^{2+}/Mg^{2+} ions but without phenol red) in order to remove non-adherent cells and any contaminating lymphocytes. After the final wash, IMDM (0.5 ml/well) supplemented with penicillin (100 $U.ml^{-1}$) and streptomycin (100 $U.ml^{-1}$) plus 10% (v:v) autologous serum. Cells were incubated (37°C; 95% O_2: 5% CO_2) for 24 h prior to experiments on monocytes. Alternatively, cells were cultured (37°C; 95% O_2: 5% CO_2) for 5 – 7 days to allow monocyte differentiation into monocyte-derived macrophages. During this 5 – 7 day incubation, the IMDM (plus supplements) was replaced on day 3 and again immediately prior to any experimental protocol.

2.2.3 Bovine Aortic Smooth Muscle Cell Culture

Commercially available bovine aortic smooth muscle cells (BAoSMC; Cell Applications Incorporated, San Diego, USA) were cultured (37°C; 95% O_2: 5% CO_2) in tissue culture flasks of increasing size (15 cm^2 up to 162 cm^2) from cyropreserved stocks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 $U.ml^{-1}$), streptomycin (100 $U.ml^{-1}$), L-glutamine (200 mM) and heat inactivated FCS (10% v:v). When a confluent monolayer of cells had formed, cells were removed from the flasks (0.25 % typsin solution with EDTA) and a cell pellet

obtained by centrifugation (350*g*; 5 min). The pellet was then re-suspended (1 ml; DMEM plus supplements) and viable cell yield obtained by trypan blue exclusion as described in section 2.2.1 and figure 2.4. Following assessment of viable cell yield, cells were then re-suspended at a density of 0.5 x 10^6 cells/ml (DMEM; plus supplements), seeded in 48 well flat-bottomed tissue culture plates at a density of 10 x 10^3 cells/well (1 x 10^3 cells/mm^2), or in 96 well flat-bottomed tissue culture plates at a density of 2.5 x 10^3 cells/well (8 x 10^2 cells/mm^2) and incubated (37°C; 95% O_2: 5% CO_2) for 24 hr (in order to allow cells to adhere to the plate) prior to the relevant experimental assay.

2.3 Assessment of Cell Viability

2.3.1 Cell Morphology

THP-1 cells were seeded as described in section 2.2.1 in 96-well round-bottomed, Flexiwell plates (BD Falcon) and incubated (37°C; 95% O_2: 5% CO_2; 2, 4, 6, and 16 h) in the absence and presence of NO donor compounds (DEA/NO, SNVP, GEA-3162; all 100 μM), or in the presence of the maximum concentration of drug vehicle (DMSO; 1%). Additionally, the known apoptotic agent, gliotoxin (1 $\mu g.ml^{-1}$), was used a positive control. Following incubation, recovered cells (100 μl) were aspirated from the 96-well plate and cyto-centrifuged (200 rpm; 2 min) in duplicate. Resultant slides were fixed in methanol (100%; 1 min) and stained using Diff-Quik™ physiological stain prior to observation by light microscopy (x 100 magnification). Apoptotic cells were identified as those with a darkly stained, condensed, pyknotic nucleus, whilst necrotic cells were identified as those with a disrupted membrane (figure 2.7). However, this assay was not considered sensitive

enough to truly distinguish between apoptosis and necrosis, so figures relating to necrotic and apoptotic cells were added together to give a total cell death count. At least 200 cells per slide were counted with the observer blinded to the experimental conditions by randomising the order of slide counting and use of opaque tape to conceal the slide label.

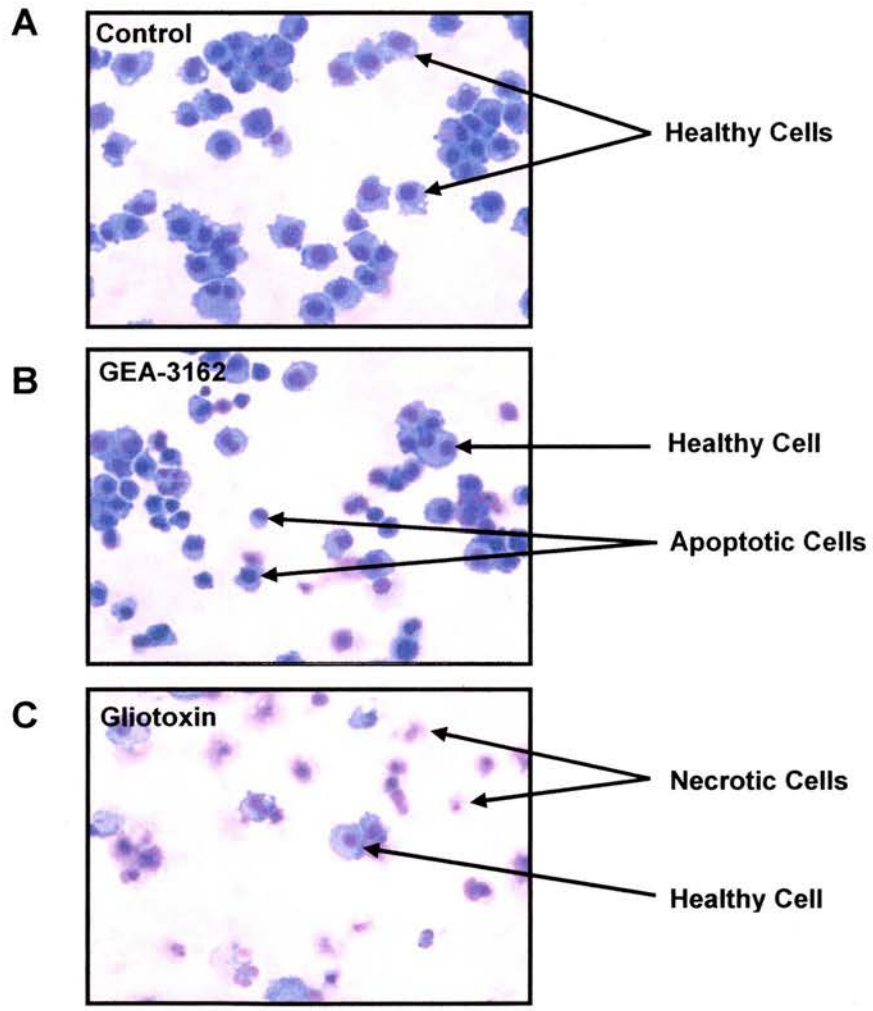

Figure 2.7 Assessment of Cell Death by Cell Morphology

Photograph (x100 magnification) demonstrating altered morphology of healthy, apoptotic and necrotic THP-1 macrophages. Cells were incubated (37 °C; 95% O_2: 5% CO_2; 16 h) in the absence of treatment (control; A) and in the presence of GEA-3162 (100 μM; B) or Gliotoxin (1 $\mu g.ml^{-1}$), prior to fixing (100% methanol) and staining (DiffQuik™ physiological stain). Apoptotic cells have a darkly stained, condensed, pyknotic nuclei compared to viable cells, whilst necrotic cells have a disrupted cell membrane.

2.3.2 Lactate Dehydrogenase Assay

The cytotoxic impact of DEA/NO, DETA/NO, SNVP, GSNO, and GEA-3162, on BAoSMC, was assessed by measuring the quantity of the enzyme lactate dehydrogenase (LDH) released from cells with a damaged plasma membrane. LDH is a stable cytoplasmic enzyme present in all cell types which is rapidly released from the cell following damage to the plasma membrane. The Cytotoxicity Detection Kit (Roche) allows quantification of the amount of LDH released from damaged cells into the culture supernatant by exploiting a two-step reaction in which LDH catalyses conversion of a tetrazolium salt to coloured formazan salt which absorbs light at 490 nm. Therefore, absorption at 490 nm is proportional to the number of damaged cells. Remaining viable cells are then lysed in order to calculate the proportion of dead cells as a percentage of the total cell population as indicated below.

$$\left(\frac{\text{Absorbance 490 mn supernatant}}{\text{Absorbance 490 mn (supernatant + lysed cells)}} \right) \text{X } 100 = \text{\% Cytotoxicity}$$

BAoSMC were seeded (2.5 x 10^3 cells/well) in 96-well, flat-bottomed microtiter plates and incubated (DMEM plus supplements; 37°C; 95% O_2: 5% CO_2) for 24 h as described in section 2.2.3. Following this initial incubation period, the DMEM was replaced and the cells incubated (37°C; 95% O_2: 5% CO_2) in the absence and presence of the each NO donor compound (10 – 300 μM) or in the presence of the maximum concentration of drug vehicle (DMSO; 1%) for a further 24 h, at the end of which the cell culture supernatants were aspirated from each well and plated in a separate 96-well, flat-bottomed, microtiter plate. LDH activity in the

supernatant was measured by determining the optical density (490 nm) following incubation (20 min; RT; light proof conditions) with the tetrazolium salt solution (at the end of the incubation period the reaction is stopped using 0.2 M HCl). Total LDH activity was assessed by lysing the remaining cells (2% Triton-X 100 prepared in DMEM) prior to determining LDH activity as above. The absorbance of each concentration of the NO donor compounds, or that of the vehicle (DMSO; 1%), was measured and subtracted from the appropriate supernatant reading, as was the absorbance of DMEM. For the cell lysis measurements, the absorbance of 2% Triton-X 100 in DMEM was subtracted from each well. All samples were assayed in duplicate. Optical densities (490 nm) were measured by a Multiskan Ascent plate reader and data captured using Ascent™ v 2.6 software (Thermo Labsystems, South Trentham, U.K).

2.3.3 Flow cytometry

In order to characterise cell death induced by DEA/NO, DETA/NO, SNVP GSNO, and GEA-3162, as either apoptosis or necrosis, flow cytometric analysis was carried out to measure the percentage of cells positive for specific cell surface markers of apoptosis and the percentage of cells with a disrupted membrane (necrotic cells). Flow cytometry is a method of characterising individual particles, or cells, based on how they fluoresce and scatter light as they are passed in suspension though a laser beam. The light scatter can be used to measure the size and granularity of a cell: forward scatter (FS) provides information on the relative size of a cell, and side scatter (SS) provides information about the relative granularity. For example, when a cell becomes apoptotic it shrinks, therefore the forward scatter decreases compared

to cells remaining viable. Additionally, cell surface changes can be detected by use of cell permeable dyes, or of fluorescent-conjugated antibodies raised against specific cell surface markers. For example, during apoptosis, phosphatiylserine (PS) translocates from the inner, to the outer, leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V – a binding protein with a high affinity for PS. Apoptosis can therefore be quantified by measuring fluorescein isothiocyanate (FITC)-conjugated Annexin V binding (Vermes et al. 1995). Alternatively, when cells undergo necrosis (or during the later stages of apoptosis) the cell membrane is disrupted, allowing dyes such as propidium iodide (PI) to penetrate the cell. Therefore, necrotic or late apoptotic cells can be identified as those positive for PI staining. Cells that are negative for both Annexin V binding and PI staining are considered healthy, viable cells. Those that are positive for Annexin V binding in the absence of PI staining are considered to be undergoing early apoptosis, whilst those that are positive for PI staining or for both Annexin V binding and PI staining are considered to have undergone necrosis or to be in the late stage of apoptosis. Annexin-V positive cells are detected in the Fl 1 channel of the flow cytometer (figure 2.6), and PI positive cells are detected in the Fl 3 channel (figure 2.6).

Human monocytes, monocyte-derived macrophages or BAoSMC were seeded in 48-well, flat-bottomed tissue culture plates as described in section 2.2.2.1 and 2.2.3 respectively. Cells were incubated (6h or 24h; 37°C; 95% O_2: 5% CO_2) in the presence and absence of each NO donor compound (100 – 300 μM for human monocyte-derived macrophges; 10 – 300 μM for BAoSMC), or in the presence of the maximum drug vehicle concentration (DMSO; 1%), or the known apoptotic agent

gliotoxin (1 $\mu g.ml^{-1}$). At the end of this incubation period, the cell culture supernatant was aspirated and discarded and the cells removed from the tissue culture plate (0.25 % typsin with EDTA). The recovered cell suspensions were removed to flow cytometry tubes and incubated (4°C; 10 min) in the presence of FITC-conjugated Annexin V in Annexin V-binding buffer (prepared as a 1:500 dilution of FITC-conjugated Annexin V in HBSS containing 5 mM $CaCl_2$). Following this incubation, PI (2 $\mu g.ml^{-1}$ final concentration) was introduced to the cell suspension/Annexin V binding buffer mix and incubated (4°C; 1 min) prior to analysis by flow cytometry. Flow cytometric analysis was carried out using a Coulter EPICS XL flow cyometer (Beckman Coulter, California, USA) and data captured using EXPO™ 32 *v* 2.1 analysis software (Beckman Coulter, California, USA).

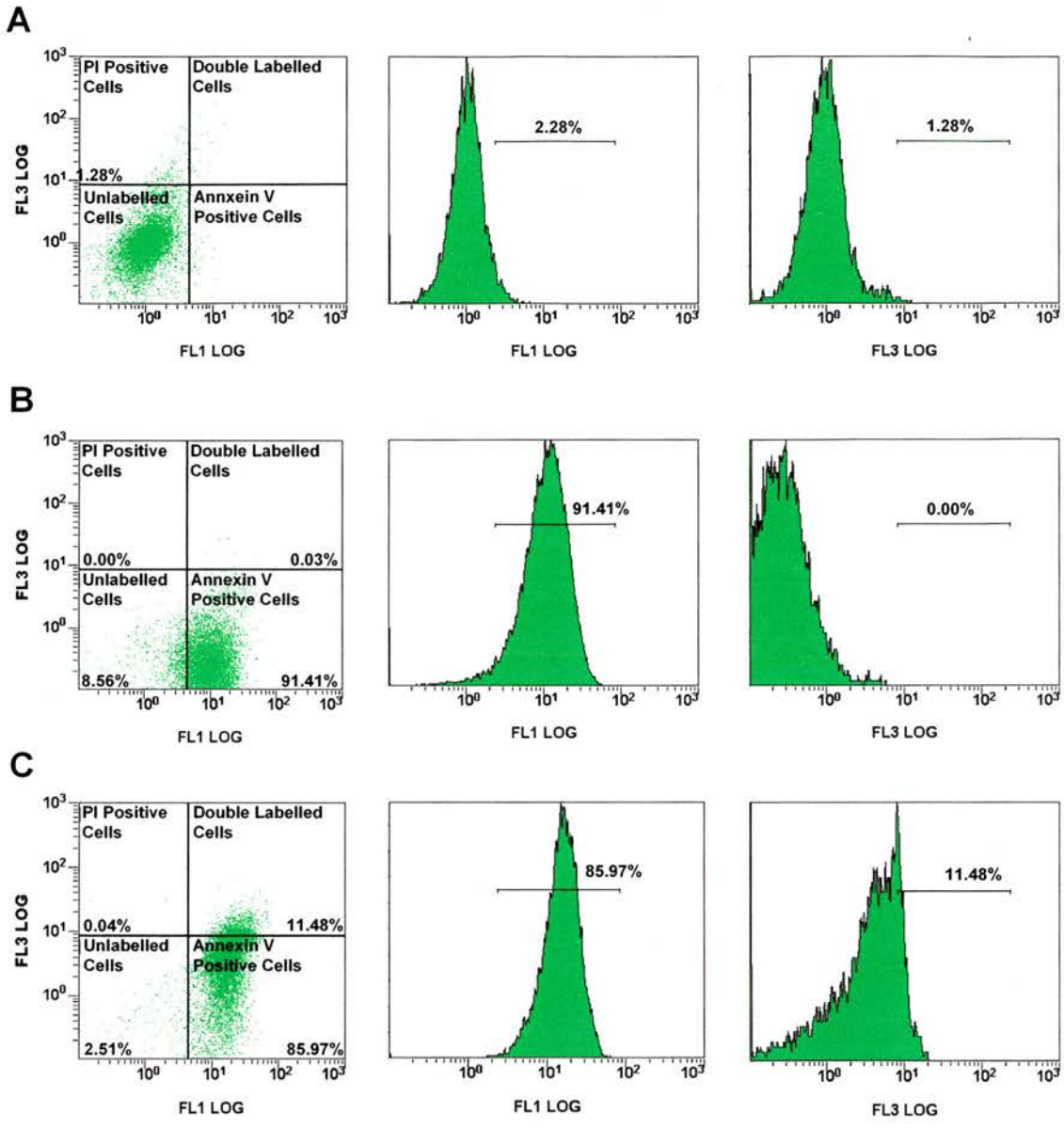

Figure 2.8 Assessment of Apoptosis and Necrosis by Flow Cytometry

Human monocyte-derived macrophages were incubated (24 h; 37°C; 95% O_2: 5% CO_2) in the absence of any treatment (A), and the presence of gliotoxin (1 µg.ml-1; B) or GEA-3162 (300 µM; C). Flow cytometry data are shown as two dimensional dot plot diagrams (left panel) and histogram plots of cells detected in the Fl 1 channel (Annexin V-positive cells; centre panel) and the Fl 3 channel (PI-positive cells; right panel).

In order to verify that a large population of apoptotic cells had not been discarded in the culture supernatant due to loss of adherence of dead cells, a sample of tissue culture supernatants was also analysed by flow cytometry. Supernatants were found to contain both healthy and apoptotic cells in low numbers, but importantly, the pattern of cell death in the supernatants correlated with that of the cell population remaining adhered to the tissue culture plate (figure 2.7).

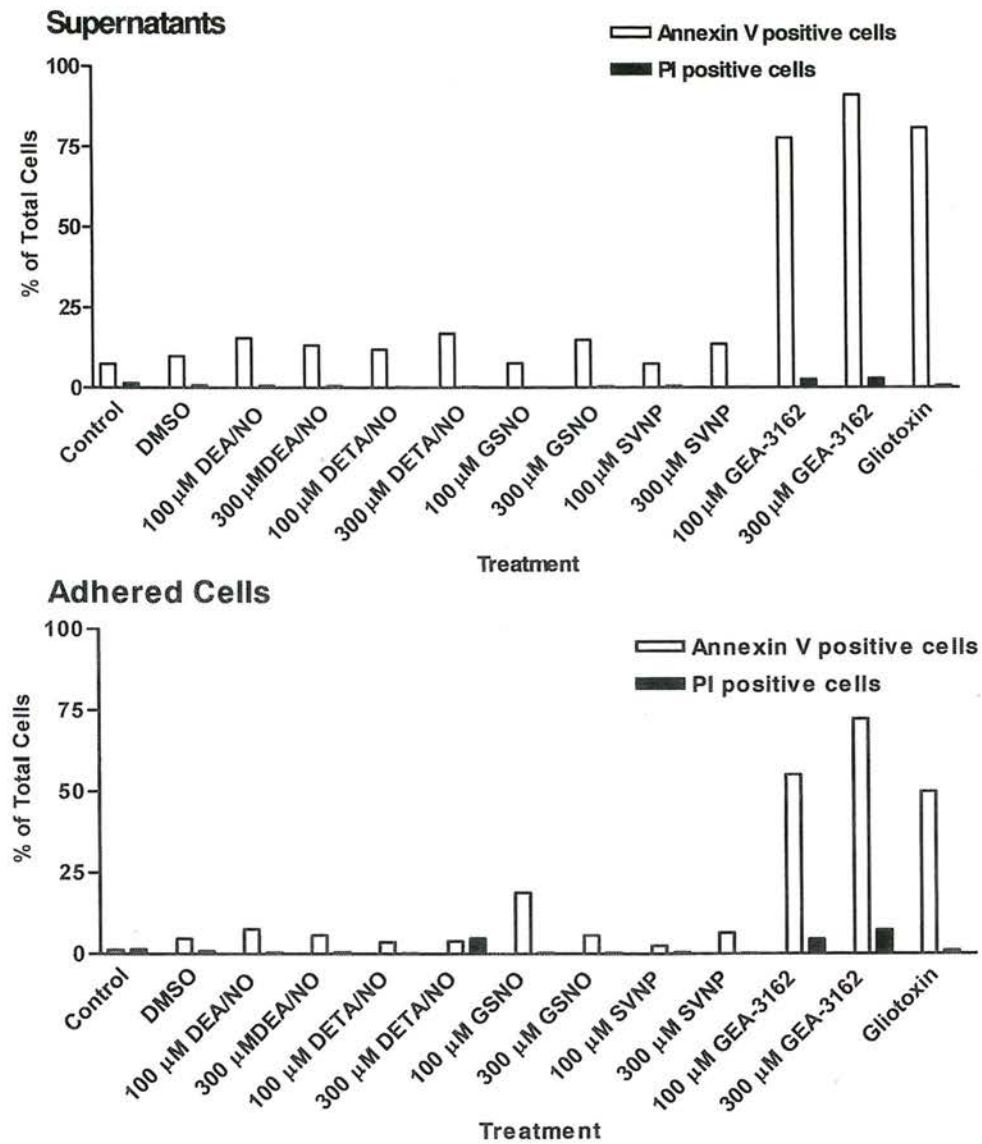

Figure 2.9 Cell Death in Human Monocyte-Derived Macrophages

Cell death in cells present in the culture supernatant is representative of those remaining adhered to the tissue culture plate (n=2).

2.4 Assessment of Cell Proliferation

Cell proliferation induced by DEA/NO, DETA/NO, SNVP, GSNO, and GEA-3162 (all 10 – 300 μM) in BAoSMC was assessed by bromodeoxyuridine (BrdU) assay. BrdU is a thymidine analogue which becomes incorporated into the newly synthesised DNA of actively proliferating cells. The BrdU Cell Proliferation Assay (Calbiochem, Merck Biosciences, Darmstadt, Germany) allows detection and quantification of BrdU, following partial denaturation of the DNA double strand by use of a detector monoclonal anti-BrdU antibody to bind to any BrdU incorporated into cells. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody is then used to bind to the detector antibody and catalyse the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) to form a coloured product that absorbs light at dual wavelengths of 450nm and 540nm. The intensity of the coloured product is directly proportional to the amount of incorporated BrdU present in the cells.

BAoSMC were seeded in 96-well, flat-bottomed microtiter plates at a density of 2.5 x 10^3 cells/well (8 x 10^2 cells/mm^2) and incubated (37°C; 95% O_2: 5% CO_2) for 24 hr to allow cells to adhere to the plate as described in section 2.2.3. This density of cells was found to be sufficient to produce approximately 70% confluence in the individual wells after the initial 24 h incubation, but was insufficient to produce 100% confluence, thereby avoiding cell-cell contact inhibition of cell proliferation. After this initial period, cells were incubated (37°C; 95% O_2: 5% CO_2) for a further 24 h in the presence of BrdU together with each NO donor compound (10 – 300 μM), or the maximum concentration of drug vehicle (DMSO; 1%). At the end of this incubation period, the cells were fixed and denatured according to the assay kit instructions prior to incubation (1 h; RT) with the anti-BrdU antibody,

followed by incubation (30 min; RT) with horseradish peroxidase-conjugated goat anti-mouse IgG. Finally, the cells were incubated (15 min; RT; light proof conditions) with TMB. After 15 min, the reaction was stopped (sulphuric acid; 2.5 M) and the optical densities (dual wavelengths 450nm and 540nm) measured by a Multiskan Ascent plate reader and data captured using Ascent v 2.6 software (Thermo Labsystems, South Trentham, U.K). The absorbance of cells without the BrdU label, and that of the tissue culture media (DMEM), were measured and subtracted from each reading. All samples were assayed in duplicate.

In order to verify that any observed reduction in cell proliferation was not due to an increased cytotoxicity resulting from the drug vehicle, DMSO, the effect on cell proliferation of the final concentrations of DMSO (0.01 – 1%) was investigated as described above. DMSO was not found to have a significant impact on the levels of cell proliferation (P>0.05; unpaired, one-way ANOVA; n=6; figure 2.8).

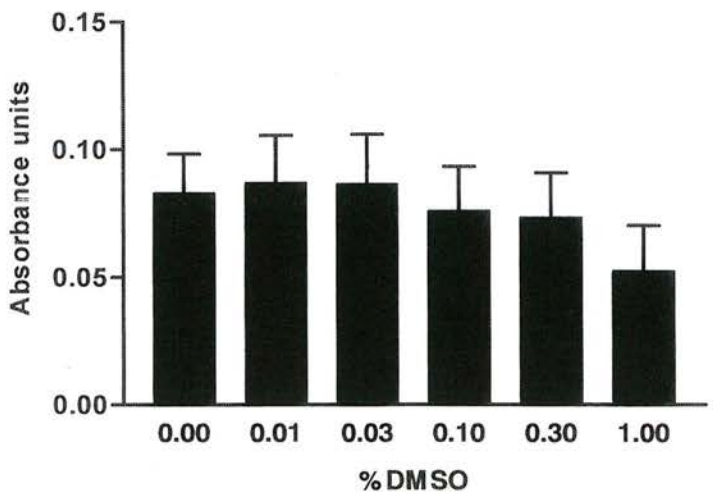

Figure 2.10 The Effect of DMSO on Cell Proliferation

DMSO (0 – 1%) has no impact on cell proliferation (*P*>0.05; unpaired, one-way ANOVA; n=6)

2.5 Measurement of cGMP

Levels of cGMP produced by human monocyte-derived macrophages and BAoSMC in response to treatment with DETA/NO (10 – 100 μM) in the absence and presence of the NO-independent sGC-stimulator, BAY 41-227 (1 μM), or GEA-3162 (100 μM) were measured by an enzyme-linked immunosorbant (ELISA) cGMP assay (R&D Systems Abingdon, UK). cGMP present in the sample competes with a pre-existing, fixed amount of cGMP for binding sites on a goat anti-rabbit polyclonal antibody coated to the microtiter plate. The level of bound enzyme is determined by addition of *p*-nitrophenol phosphate (pNPP) substrate, which produces a coloured product that absorbs light at 405 nm. The intensity of the colour produced is inversely proportional to the amount of bound antibody. Low levels of cGMP are measured by acetylation of the sample with acetic anhydride and triethylamine at low pH.

Human monocyte-derived macrophages or BAoSMC were seeded in 48-well, flat-bottomed tissue culture plates as described in section 2.2.2.1 and 2.2.3 respectively. Cells were incubated (37°C; 95% O_2: 5% CO_2; 24 h) in the presence of NO-donor compounds plus the non-specific phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX; 250μM) to prevent cGMP breakdown. Following this incubation, the cell culture supernatants were aspirated from the wells, frozen immediately on dry ice and stored (-70°C). Adherent cells were lysed (300 μl/well Triton X 100 (2%) prepared in phenol red-free HBSS containing Ca^{2+}/Mg^{2+}) and the recovered solution divided into two aliquots of 225 μl for the cGMP assay and 75 μl for a protein assay (see section 2.6 below). The aliquots for the cGMP assay were acidified (HCl; 0.1 M) and then both aliquots frozen

immediately on dry ice and stored (-70°C) for approximately one week prior to performing the assay.

Each sample was acetylated according to the cGMP assay kit instructions prior to incubation (2h; horizontal orbital shaker (500 ± 50 rpm); RT) with the cGMP conjugate and the cGMP antibody. At the end of this time, the substrate pNPP was added to the wells and the plate incubated for a further hour (RT) before the reaction was stopped (trisodium phosphate) and the absorbance (405 nm) measured. Optical densities (405 nm) were measured by a Multiskan Ascent plate reader and data captured using Ascent™ v 2.6 software (Thermo Labsystems, South Trentham, U.K). Non-specific binding (NSB) was determined by addition of the cGMP conjugate and cGMP antibody to wells coated with the goat anti-rabbit polyclonal antibody in absence of any sample and this absorbance value subtracted from the absorbance of all samples. The concentration of cGMP was calculated from a standard curve produced daily from serial dilutions of known concentrations of cGMP (0.08 – 50 pmol.ml^{-1}; figure 2.9). Standards were acetylated as described above for the samples. All standards and samples were assayed in duplicate.

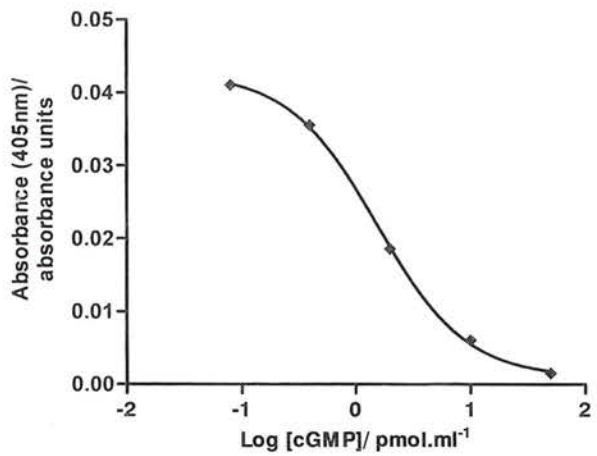

Figure 2.11 Standard Curve Generated for the Calculation of cGMP Concentration

Example standard curve generated from the absorption (405 nm) of known concentrations of acetylated cGMP (0.08 – 50 pmol.ml^{-1})

2.6 Measurement of Protein

In order to account for variability in the densities of the cell populations seeded in tissue cultures plates, the total amount of protein present in each well was measured by bicinchoninic acid (BCA) colorimetric protein assay (Pierce, Illinois, USA). During this assay, Cu^{2+} is reduced to Cu^{+} by protein in an alkaline medium (the biuret reaction). Cu^{+} ions are then detected by the reaction of the assay substrate containing BCA to form a coloured product that absorbs light at 562 nm. Aliquots of human monocyte-derived macrophages and BAoSMC cell lysates (see section 2.5) were plated out in 96-well, flat-bottomed microtiter plates. Cell lysates were incubated (37°C; 30 min) in the presence of the assay substrate containing BCA prior to determining the absorbance (562 nm) of each sample. Optical densities were determined using a Dynatech MRX plate reader (Dynatech Laboratories Incorporated, Virginia, USA). Total protein concentration of each well was calculated from a calibration graph generated daily from the absorbance (562 nm) of known concentrations of bovine serum albumin (BSA; 0 - 1000 $\mu g.ml^{-1}$) diluted in Triton X 100 (2%; prepared in phenol red-free HBSS containing Ca^{2+}/Mg^{2+}), with the curve fitted by first order polynomial nonlinear regression (figure 2.12). The absorbance of the Triton X 100 solution was recorded as a blank and this value subtracted from the readings of all standards and samples. All standards and samples were assayed in duplicate.

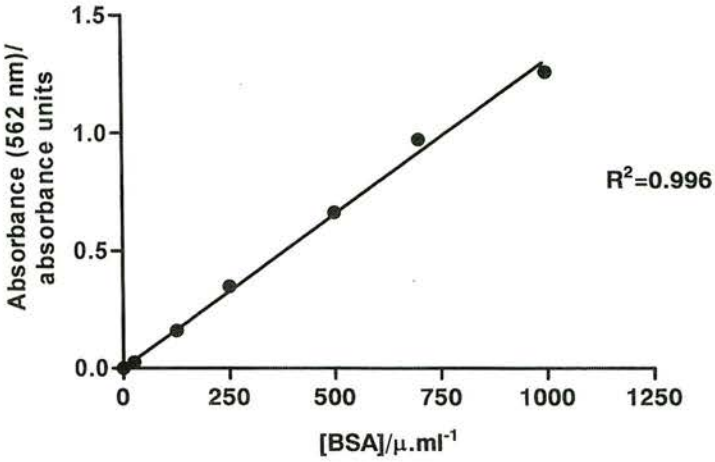

Figure 2.12 Standard Curve Generated for the Calculation of Total Protein in Cell Lysates

Example calibration graph generated from the absorbance (562 nm) of known concentrations of bovine serum albumin (BSA; 0 – 1000 $\mu g.ml^{-1}$).

2.7 Functional Studies in Isolated Rat Aortae

To test the efficacy of DETA/NO, the NO-independent sGC stimulator, BAY 41-2272, and the non-specific PDE inhibitor, IBMX, relaxation in response to each compound was measured as isometric tension in isolated rat aortic rings using a fixed-mount myogragh.

Male Wistar rats (Charles River) were housed and terminated in accordance with the Animals (scientific Procedures) Act 1986 (UK Home Office). Adult male rats were terminated by cervical dislocation and thoracic aortae harvested into physiological solution (PSS; 4°C) containing (mM) NaCl (119); KCL (4.7); $CaCl_2.2H_2O$ (2.5); $MgSO_4.7H_2O$ (1.17); $NaHCO_3$ (25); KH_2PO_4 (1.18); EDTA (0.027) and glucose (5.5). Aortae were cleaned of connective tissue and fat in PSS

(4°C), before being divided into aortic ring sections (approximately 3 mm). Aortic rings were suspended between two intraluminal wires in four parallel myograph chambers (10 ml) containing oxygenated (95% O_2: 5% CO_2) PSS (37°C). In order to model intraluminal pressure generated *in vivo*, tension was applied to each vessel in stepwise increments to obtain a resting tension of 17 mN. This isometric tension was measured in a Multi Tissue Bath System 700MO (Danish Myo Technology, Copenhagen, Denmark) and data captured via a MabLab/4e analogue-digital converter and displayed through Chart™ *v* 3.6/s software (AD Instruments, Sussex, UK).

Prior to each experimental protocol, maximum receptor-independent contraction in response to potassium (K^+) was obtained by contracting each vessel three times with high potassium PSS (KPSS) containing 123.7 mM KCl. Subsequently, cumulative concentration-response curves fitted by second order polynomial nonlinear regression were obtained in each vessel for phenylephrine (PE; 0.001 – 1 μM) and a suitable concentration selected to produce ~80% contraction (EC_{80}; 0.1 μM; figure 2.11). Following precontraction with EC_{80} PE (0.1 μM), responses to DETA/NO (0.001 – 100 μM) and DETA/NO (10 μM) in the presence of BAY 41-2272 (1 μM) or IBMX (1 μM) were measured. Contractions are expressed as a percentage of the maximum contraction in response to KPSS and relaxations as a percentage of the contraction in response to EC_{80} PE. Data are expressed as the average of four aortic rings for each animal.

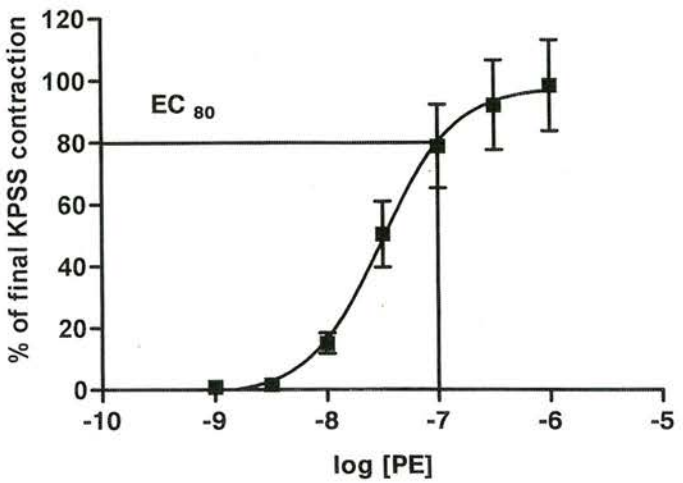

Figure 2.13 Determining the EC_{80} for Phenylephrine

Isometric tension in response to PE (0.001 – 1μM) was measured in isolated rat aortic rings and the concentration of PE giving 80% of maximum contraction selected as the EC_{80} PE. Contractions are expressed as a percentage of the maximum contraction in response to KPSS. Data are expressed as mean ± SEM, (n=4)

2.8 Statistics

Statistical analyses are stated in experimental chapters. Data were analysed using GraphPad Prism software v 3.03 (GraphPad Software Inc., San Diego, USA). Degrees of significance are abbreviated throughout as follows: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$ or by symbols as indicated in experimental chapters. In all cases, $P > 0.05$ was not considered to be statistically significant (ns; not significant). Data are expressed throughout as mean ± standard error of the mean (SEM).

2.9 Materials

NO Donor Compounds

GEA-3162, SNVP, GSNO and BAY 41-2272 were prepared and stored (-20°C) as stock solutions (10^{-1} M) in DMSO. Immediately prior to use, these compound were diluted in PBS. DEA/NO and DETA/NO were prepared and stored (-20°C) as stock solutions (10^{-2} M) in NaOH (0.01 M), and diluted prior to use in PBS. Additionally, an appropriate volume of DMSO was added to each concentration of DEA/NO and DETA/NO such that the final experimental concentration of DMSO was equal for all NO donor compounds and not greater than 1%, which was experimentally inert, thus removing the vehicle as a source of variability.

GEA-3162, DEA/NO, and DETA/NO were purchased from Axxora (Axxora Ltd, Nottingham, UK). GSNO was purchased from Sigma (Sigma Aldrich, Poole, UK). SNVP was synthesised by a previously published method (Megson et al. 1997) and kindly supplied by Drs A.R Butler and N.P Botting, University of St Andrews (St Andrews, UK). BAY 41-2272 was kindly supplied by Bayer (Leverkusen, Germany).

Met-Hb was reduced to the ferro-form with sodium dithionite as previously described (Martin et al. 1985), and aliquots (1 mM) stored (-20°C) for less than one month.

Tissue Culture

Tissue culture materials were supplied as follows:

Becton Dickinson Falcon (Oxford, UK)

All tissue culture plasticware including tissue culture flasks (15 – 162 cm^2), microtiter (96-well) plates, multi-well tissue culture plates (48- & 96-well) and 96-well Flexiwell™ plates were supplied sterile.

Gibco Life Technologies Paisley, UK)

Tissue culture media (RPMI 1640; IMDM; DMEM) were supplied sterile, endotoxin free, at pH 7. Trypsin (0.25%) EDTA solution and tissue culture supplements penicillin/ streptomycin solution and L-glutamine solution were stored (-20°C) in aliquots. Foetal calf serum was heat inactivated by heating (50°C; 1 h) in a sterile water bath before addition to media.

PBS (1x and 10x) and HBSS both with and without Ca^{2+}/Mg^{2+} as required, were supplied sterile, endotoxin free, at pH 7.

All tissue culture solutions were stored (4°C) and pre-warmed (37°C) in a sterile water bath prior to use.

Pharmacia (Milton Keynes, UK)

Percoll was stored (4°C) as 100% solution and diluted to 90% solution immediately prior to use in 10x PBS (without Ca^{2+}/Mg^{2+}). 90% Percoll solution was further diluted as required in 1x PBS (without Ca^{2+}/Mg^{2+}). Dextran T500 was stored (4°C) in aliquots.

Additional Reagents

Additional reagents were supplied and prepared as follows:

Sigma Aldrich (Poole, UK)

SOD was prepared (5000 $U.ml^{-1}$ in PBS) and stored (-20°C) in aliquots. IBMX was prepared (10^{-2} M in DMSO), stored in aliquots (-20°C) and diluted immediately prior to use in PBS. LPS was prepared (1 $mg.ml^{-1}$ in PBS) and stored (-20°C) in aliquots. DAN was prepared freshly immediately prior to use in HCl (0.7 M). Gliotoxin was prepared (1 $mg.ml^{-1}$ in DMSO), stored (-20°C) in aliquots and diluted prior to use in PBS (to give a final DMSO concentration equal to that of the NO donor compounds). NADPH was prepared freshly immediately prior to use in PBS. Nitrate reductase was prepared (10 $U.ml^{-1}$ in PBS) and stored in aliquots (-20°C). FITC- conjugated Annexin V was stored (-20°C) in aliquots and diluted prior to use in Annexin V binding buffer (prepared as HBSS plus 5 μM $CaCl_2$). PI was prepared (1 $mg.ml^{-1}$ in H_2O) and stored (4°C) in aliquots. Trypan Blue was stored at room temperature.

Fisher Scientific (Loughborough, UK)

Triton X 100; DMSO.

General laboratory reagents including buffer salts; EDTA; NaOH; HCl.

Ultrafilter centrifuge tubes (12 kD pore size)

Axxora Ltd (Nottingham, UK)

Tempone-H was prepared (0.1 M in 10 mM in EDTA solution) and stored (-20°C) in aliquots. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide .

potassium salt (carboxyPTIO) was prepared (10^{-3} M in PBS) immediately prior to use.

Tocris Cookson (Bristol, UK)

ODQ was prepared (10^{-2} M in DMSO) and stored (-20°C) in aliquots.

Rathburn (Walkerburn, UK)

HPLC-grade reagents: H_2O; Acetonitrile; NaOH; HCl

Charles River (Kent, UK)

Wistar male rats were housed and terminated according to The Animals (Scientific Procedures) Act 1986 (UK Home Office).

British Oxygen Company (Surrey, UK)

Compressed gas (95% O_2: 5% CO_2).

Chapter Three

Characterisation of NO Donor Compounds

3. Characterisation of NO Donor Compounds

3.1 Introduction

NO is often reported to have paradoxical actions in many biological settings. For example, the literature contains numerous reports of NO being both antioxidant and pro-oxidant (Goss et al. 1995; Rubbo et al. 1995; Struck et al. 1995; Joshi et al. 1999; Wei et al. 2000), cytotoxic and cytoprotective (Polte et al. 1997; Stefanelli et al. 1999; Mason et al. 2000; Keira et al. 2002; Hattori et al. 2004) and having both pro- and anti-apoptotic characteristics (Ward et al. 2000; Kim et al. 2001; Fiscus et al. 2002). Such apparent contradictions may be explained, at least in part, by the ease with which the NO radical forms intermediary, NO-related species *in vivo*. NO-related species can be biologically active independent of the liberation of NO *per se* and, therefore, the net outcome of any NO-induced response will be critically dependent on the concentration and precise NO-related species generated in the microenvironment, as well as the nature of the target cell.

Numerous synthetic NO donor compounds are available both for possible use in the clinical setting and as experimental tools. Each class of compound has unique chemical properties and varying mechanisms of NO release, ranging from compounds capable of spontaneously liberating NO radical in solution to those requiring more complex enzymatic degradation, or the presence of tissue thiol groups (Megson 2000). Such differences can be exploited *in vitro* to examine the precise NO-related species responsible for mediating particular biological actions of NO.

The diazeniumdiolate class of NO donor are considered 'pure' NO donors and are capable of releasing up to two molecules of NO spontaneously in solution without the requirement for enzymatic or tissue-dependent activation (Smith et al. 1996; Megson 2000; Megson and Webb 2002). NO is released predictably and by first order kinetics with the rate of release dependent on the nature of the nucleophile to which the [N(O)NO] group is attached, as well as temperature and pH (Davies et al. 2001). Varying the nucleophilic adduct in the molecule has produced a variety of compounds in which the rates of decomposition range from seconds to many hours (Megson 2000; Megson and Webb 2002). This makes the diazeniumdiolates particularly useful experimental tools because compounds can be selected on the basis of their individual half-lives in order to deliver NO over a range of concentrations and time periods. For example, a compound with a short half-life, such as DEA/NO ($t_{½}$=2 min), can be used to deliver a short burst of NO, whereas those with longer half-lives, such as DETA/NO ($t_{½}$=20 h), can be used to provide a prolonged and relatively sustained delivery of NO.

The S-nitrosothiol (RS-N=O) class of NO donors are formed both endogenously and synthetically by nitrosation (transfer of NO^+) of reduced cysteine residues of proteins, or thiol groups of low molecular weight compounds (Butler et al. 1995; Kharitonov et al. 1995; Butler and Rhodes 1997; Liu et al. 1998); examples include endogenous GSNO and the synthetic compound, SNVP. The biological half-life of S-nitrosothiols is unpredictable in solution and dependent on the R group of the compound (Mathews and Kerr 1993). The exact mechanism of decomposition and subsequent biological action of S-nitrosothiols, both endogenous and synthetic, is not fully known and may occur without the liberation of NO *per se* (Kowaluk and

Fung 1990; Ceron et al. 2001), but via rapid transnitrosation reactions resulting in the transfer of NO^+ from one thiol moiety to another (Scharfstein et al. 1994; Askew et al. 1995; Liu et al. 1998; Hogg 1999). However, release of free NO from S-nitrosothiols does occur when the rate of decomposition is greatly accelerated due to the presence of copper ions or exposure to light (Sexton et al. 1994; Askew et al. 1995; Gordge et al. 1995; Dicks and Williams 1996; Gorren et al. 1996; Singh et al. 1996; Al-Sa'doni et al. 1997).

Mesoionic 3-aryl, 5-imino-oxatriazole derivatives, for example GEA-3162, are a novel class of NO donors which are structurally very similar to the sydnonimines (Megson 2000; Megson and Webb 2002). Sydnonimines, for example SIN-1, are not strictly NO donors but are considered generators of the NO-related species, $ONOO^-$, on account of concomitant generation of NO and O_2^-, which rapidly combine to form $ONOO^-$ (Feelisch et al. 1989). Early investigations with the mesoionic 3-aryl-substitued oxatriazole-5-amine derivatives initially reported this class of compound to be pure NO donors (Kankaanranta et al. 1996; Holm et al. 1998). However, recent evidence has suggested that, in common with the structurally similar compound SIN-1, GEA-3162 is in fact a $ONOO^-$ generator, rather than an NO donor (Taylor et al. 2004).

In order to use this variety of NO donor compounds as useful experimental tools, it is essential to characterise precisely which NO-related species is generated during an assay. Because the profile of NO release and rate of decomposition from such compounds depends on the composition of the environment into which they are introduced (e.g. pH, temperature), it is prudent to characterise the NO-related species generated by each compound in conditions as close as possible to those prevalent

during subsequent experimental assays. This is particularly true for cell culture experiments as components of the tissue culture medium may affect NO (Keynes et al. 2003). Therefore, the aim of these studies was to fully characterise the NO, or NO-related species, generated under conditions used during subsequent biological investigations involving the diazeniumdiolates, DEA/NO and DETA/NO; the S-nitrosothiols, SNVP and GSNO; and the mesoionic 3-aryl-substitued oxatriazole-5-amine derivative, GEA-3162.

1.1 3.2 Methods

3.2.1 Electrochemical Detection of NO

NO released in solution was measured using a calibrated isolated NO electrode (see chapter 2.1.1). The NO donor compounds DEA/NO (10 μM), DETA/NO (300 μM), SNVP (300 μM) and GSNO (300 μM) were added in the presence or absence of SOD (500 $U.ml^{-1}$) to aliquots (2 ml) of pre-warmed (37 °C), stirred (600 rpm) DMEM plus supplements (as described in chapter two, section 2.2.3) or IMDM plus supplements but without serum (see chapter two, section 2.2.2.1), and the signal from the electrode recorded for 30 minutes (figures 3.1 & 3.3). For those experiments conducted in the presence of SOD, the signal from the NO electrode was recorded for 1 min in the presence of SOD (500 $U.ml^{-1}$) alone prior to the addition of NO donor compounds. At the end of this time, the NO scavenger, Hb (5 μM), was introduced to the electrode chamber. GEA-3162 (300 μM) was added to aliquots of pre-warmed (37 °C), stirred (600 rpm) DMEM or IMDM and the signal recorded for 1 min prior to the introduction of stepwise increments of SOD (50 – 500 $U.ml^{-1}$). When a final concentration of 500 $U.ml^{-1}$ had

been reached, Hb (5 μM) was introduced to the electrode chamber. Alternatively, GEA-3162 (300 μM) and SOD (500 $U.ml^{-1}$) were added simultaneously to aliquots of pre-warmed (37 °C), stirred (600 rpm) DMEM or IMDM and the NO release recorded until the signal had decayed to baseline.

The above recordings were also performed in H_2O buffered with sodium bicarbonate ($NaHCO_3$) to mirror the buffering of DMEM or HEPES to mirror that of IMDM (see appendix one), and in IMDM containing heat inactivated FCS (10% v:v) in addition to the supplements described in chapter two (see section 2.2.2.1).

3.2.2 Electron Paramagnetic Resonance

Oxidising free radical species generated by each NO donor compound (10 – 300 μM) were measured in DMEM and IMDM in presence and absence of SOD (500 $U.ml^{-1}$) using the chemical spin-trap, Tempone-H, as described in chapter 2.1.4. Only those signals where the signal-to-noise ratio was >3 were considered to genuine signals. Despite preparation of the spin trap in EDTA (10 mM) to minimise the phenomenon, relatively small signals were generated in control experiments (Tempone-H (1 mM) in each medium) due to auto-oxidation of the spin trap. These signals have been subtracted from the corresponding experimental signals detected in the presence of NO donor compounds.

3.3 Results

3.3.1 NO Electrode Studies

NO release from DEA/NO (10 μM) was rapid and short-lived in both media, reaching a maximum signal by approximately 5 min in DMEM and 2.5 min in IMDM, with the signal decaying to baseline by approximately 30 min in both media (figures 3.1 & 3.3). In DMEM, a maximum concentration of 11.2 ± 2.2 μM NO was attained from DEA/NO, and this was unaffected by the presence of SOD (500 $U.ml^{-1}$), when the peak concentration was 11.1 ± 1.8 μM (figure 3.3 & table 3.1). Analysis of the area under the curve (AUC; figure 3.3) by unpaired, two-tailed t-test revealed this difference to be non-significant ($P>0.05$; n=6). Similarly, in IMDM, the peak NO concentration attained from DEA/NO was 8.9 ± 1.0 μM (figure 3.3 & table 3.1), and this was not significantly affected by SOD (10.3 ± 0.6 μM; $P>0.05$; unpaired, two-tailed t-test of AUC; n=6). Analysis of AUC of DMEM compared to IMDM (comparison of the graphs in figure 3.3) revealed the release of NO from DEA/NO was not significantly altered between the two media, both in the absence and presence of SOD ($P>0.05$; unpaired, two-tailed, *t*-test AUC; n=6; table 3.1).

DETA/NO (300 μM) produced a slower, more prolonged NO release in both media, with the maximum signal reached at approximately 10 min, and then remaining steady for the duration of the 30 min recording (figure 3.2 & 3.3). Hb (5 μM) completely abolished the signal from DETA/NO at the end of the 30 min recording (figures 3.2 & 3.3). The release of NO from DETA/NO was unaffected by SOD (500 $U.ml^{-1}$) in both media (maximum concentrations of 3.9 ± 0.1 μM *vs* 4.1 ± 0.4 μM; $P>0.05$ for DMEM and 2.6 ± 0.2 μM *vs* 2.6 ± 0.2 μM; $P>0.05$ for IMDM, unpaired, two-tailed *t*-test of AUC; n=6 for both media; figure 3.3). However,

analysis of AUC (figure 3.3) by unpaired, two-tailed *t*-test revealed the total amount of NO released from DETA/NO was significantly greater in DMEM compared to IMDM over the 30 min incubation period, both in the absence ($P < 0.01$) and presence of SOD ($P < 0.05$; table 3.1).

The S-nitrosothiols, SNVP and GSNO (both 300 μM), released only low concentrations of NO in solution (figures 3.4 – 3.6). SNVP released significantly more NO in DMEM compared to IMDM (maximum concentrations of 2.4 ± 0.8 μM *vs* 0.4 ± 0.1 μM; $P < 0.01$; unpaired, two-tailed t-test of AUC; table 3.1). Hb (5 μM) abolished the signal from SNVP when it had not already decayed to base line by the end of the 30 min recording (figures 3.4 & 3.6). The presence of SOD significantly increased the release of NO from SNVP in both DMEM ($P < 0.05$) and IMDM ($P < 0.001$; unpaired, two-tailed *t*-test AUC; n=6; for both media; figure 3.6). Similarly, GSNO released significantly more NO in DMEM compared to IMDM (maximum concentrations of 5.1 ± 0.2 μM *vs* 2.0 ± 0.2 μM; $P < 0.001$; unpaired, two-tailed, *t*-test AUC; n=6; table 3.1). Hb abolished the signal from GSNO at the end of the 30 min recording (figures 3.5 & 3.6). The presence of SOD did not significantly affect the amount of NO released from GSNO in either media (for both media $P > 0.05$; unpaired, two-tailed, *t*-test AUC; n=6; figure 3.6).

Both SNVP and GSNO, regardless of the absence or presence of SOD, generated a biphasic NO release profile consisting of a rapid immediate peak, which then decayed superimposed on a second slower and more sustained phase of NO release. This occurred in both media, but was more pronounced for GSNO than SNVP and more marked in DMEM than IMDM (figures3.4 – 3.6)

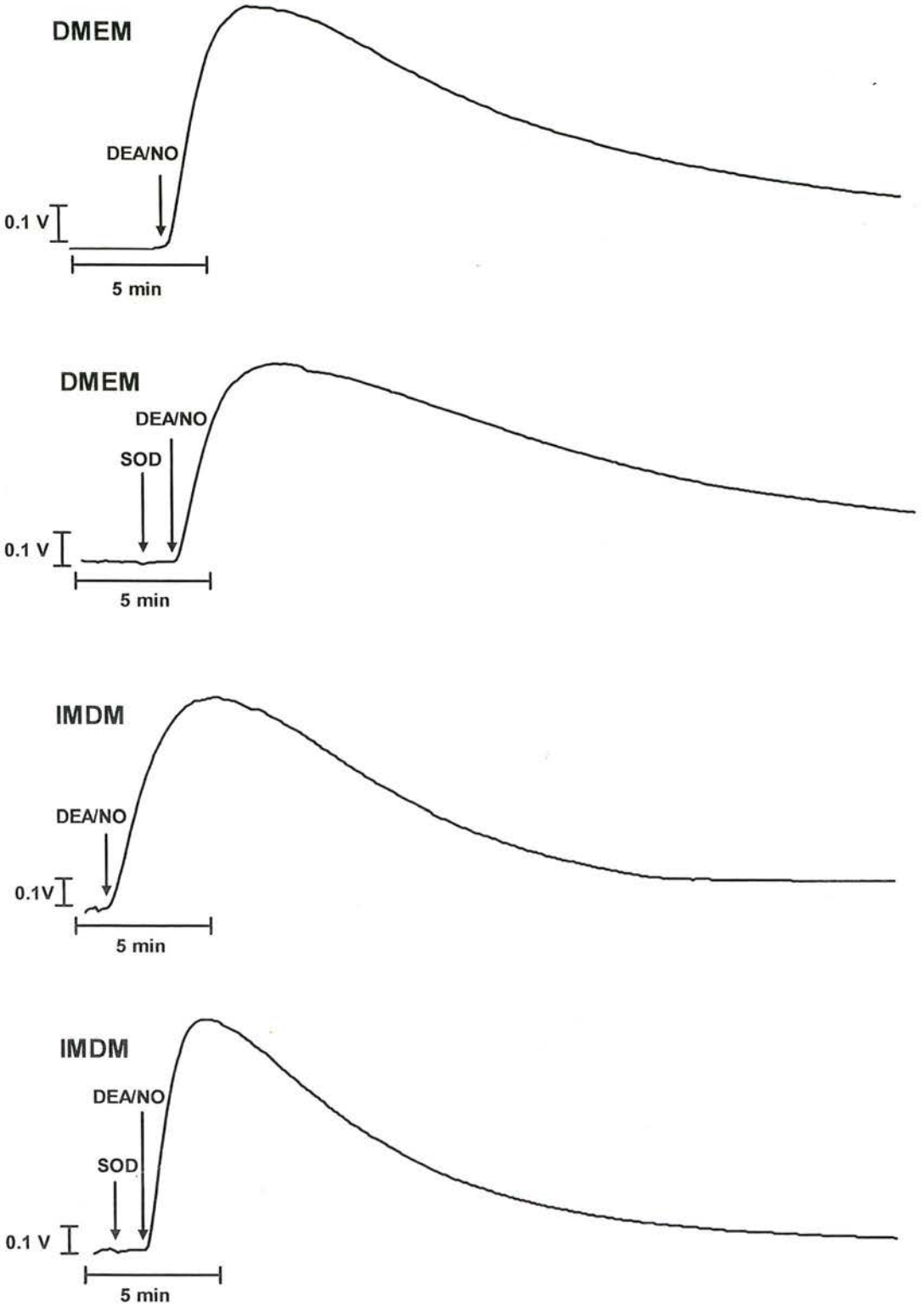

Figure 3.1 NO Release from DEA/NO in DMEM and IMDM

Example traces from the NO electrode showing NO release from DEA/NO (10 μ) in DMEM and IMDM in the absence and presence of SOD (500 $U.ml^{-1}$). Arrows show addition of compounds as described indicated.

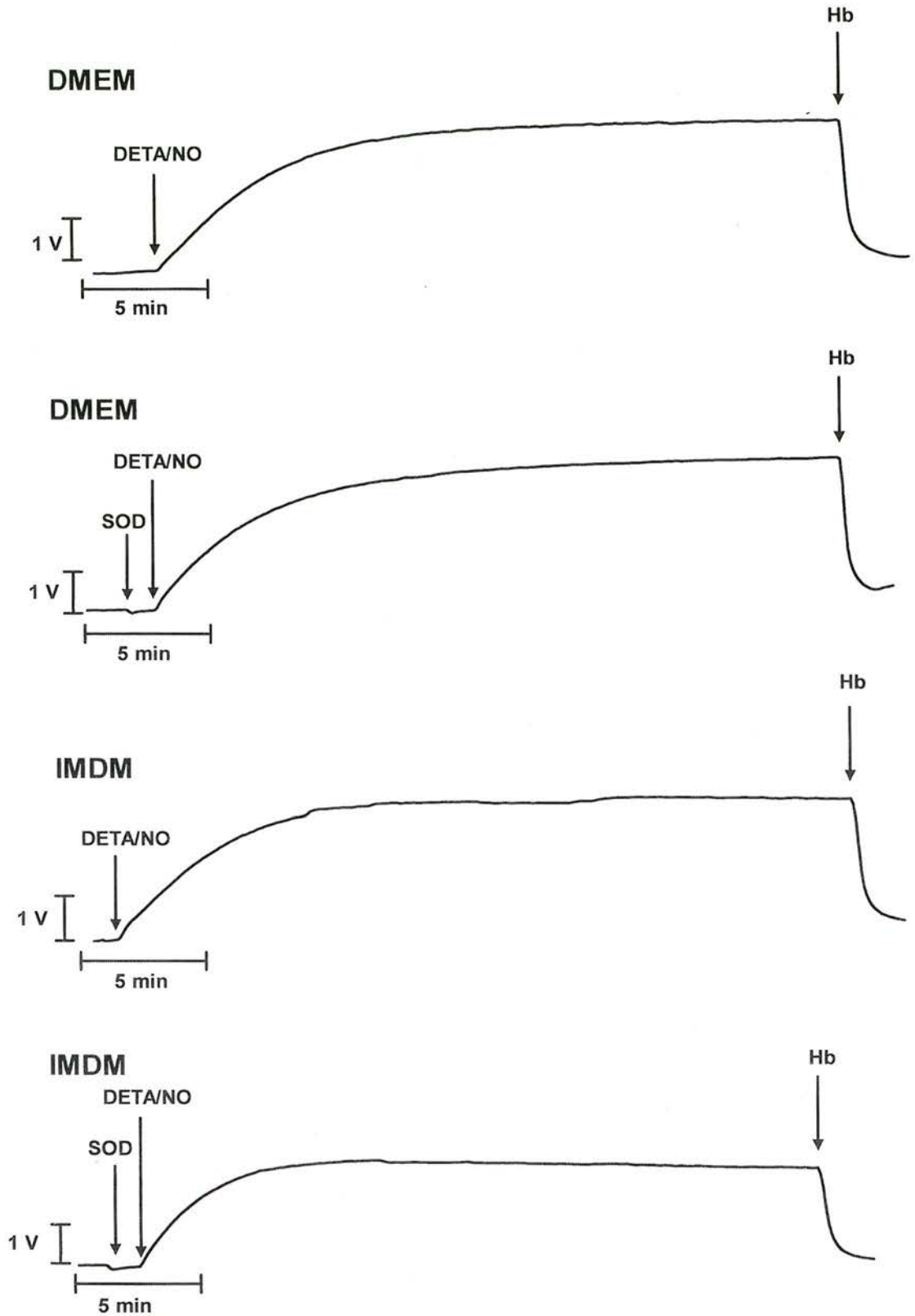

Figure 3.2 NO Release from DETA/NO in DMEM and IMDM

Example traces from the NO electrode showing NO release from DETA/NO (300 μM) in DMEM and MDM in the absence and presence of SOD (500 $U.ml^{-1}$). The signal in all cases was abolished by addition of Hb (5 μM). Arrows show addition of compounds as indicated.

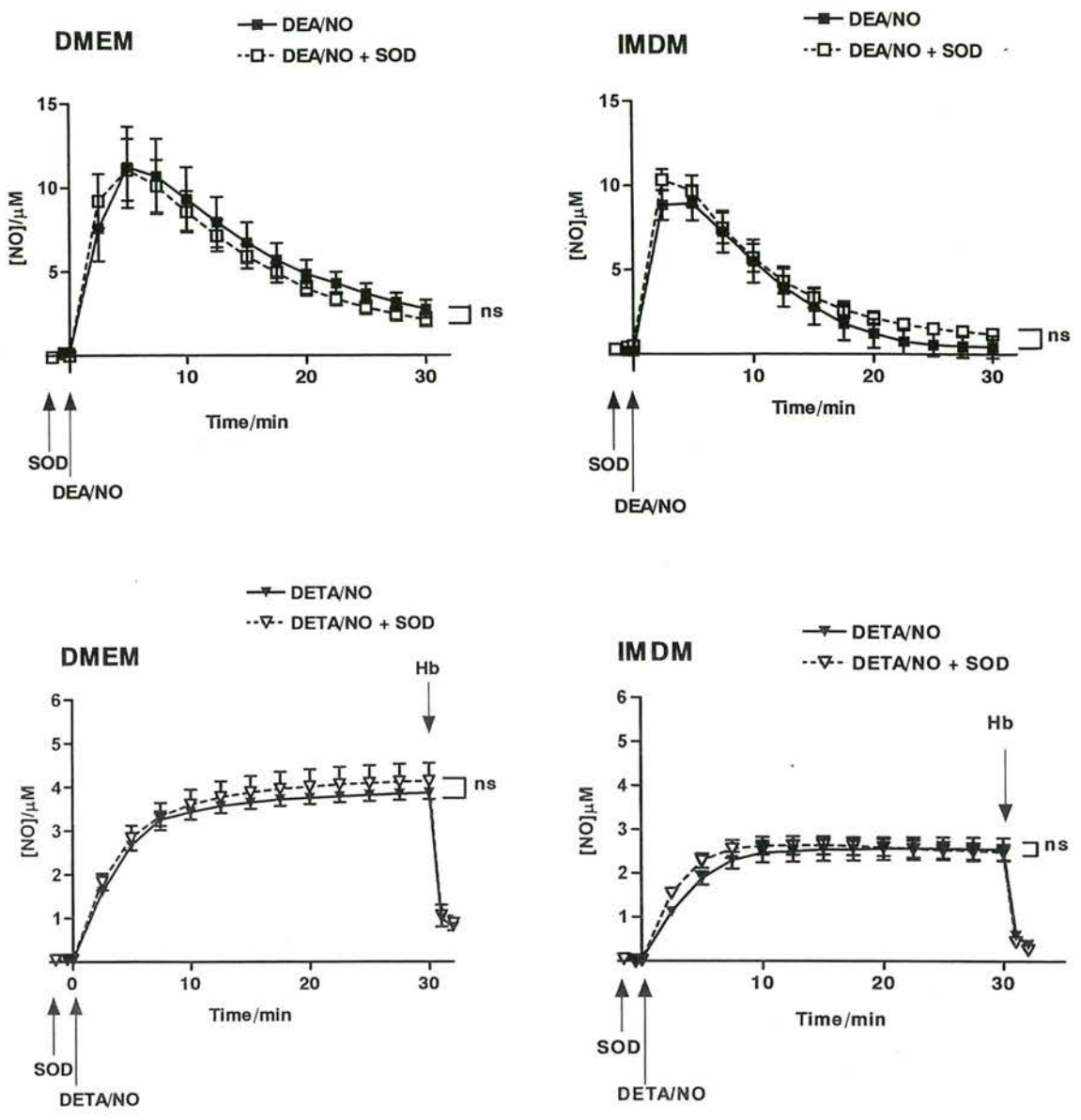

Figure 3.3 NO Release from DEA/NO and DETA/NO

NO release from DEA/NO (10 μM) and DETA/NO (300 μM) was measured by calibrated isolated NO electrode in DMEM (left panel) and IMDM (right panel) in the absence (closed symbols, solid lines) and presence of SOD (500 $U.ml^{-1}$; open symbols, dashed lines). Hb (5 μM) abolished the signal from DETA/NO in both media. Arrows show addition of compounds as indicated. Analysis of the area under the curve (AUC) by unpaired, two-tailed *t*-test revealed SOD did not significantly affect NO release (ns = $P>0.05$; n=6).

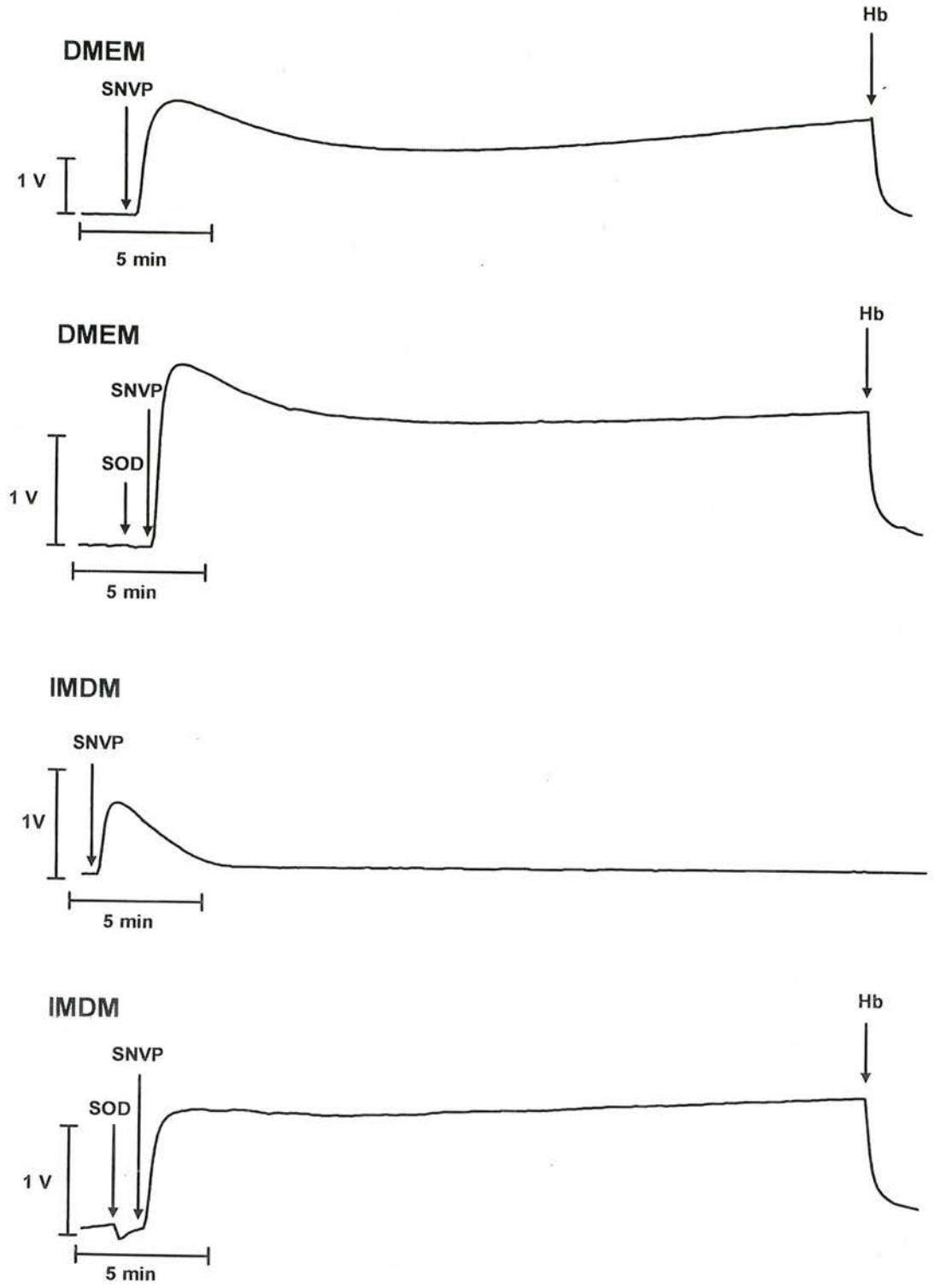

Figure 3.4 NO Release from SNVP in DMEM and IMDM

Example traces from the NO electrode showing NO release from SNVP (300 μM) in DMEM and IMDM in the absence and presence of SOD (500 $U.ml^{-1}$). Hb (5 μM) abolished the signal when it had not already decayed to baseline. Arrows show addition of compounds as indicated.

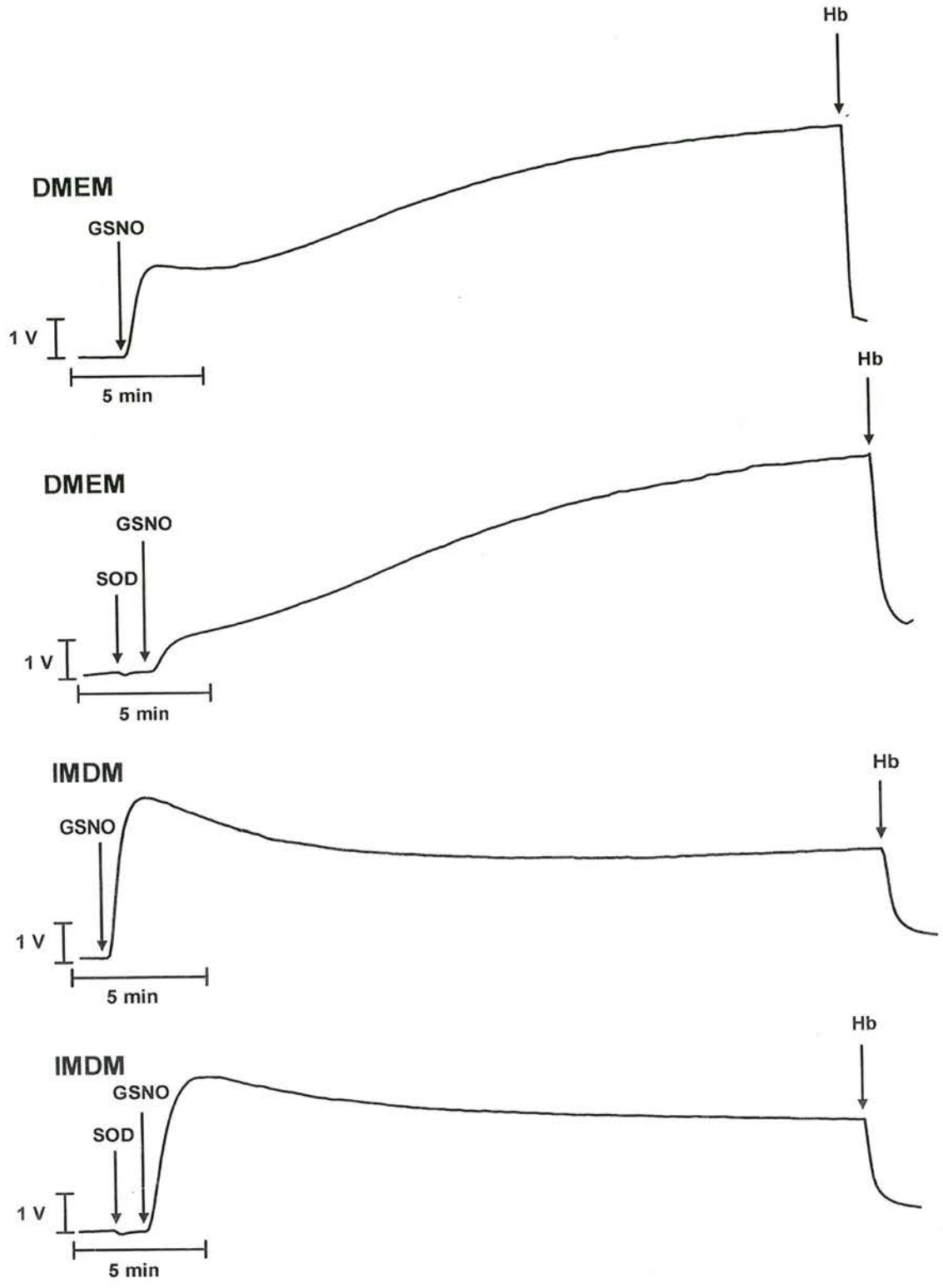

Figure 3.5 NO Release from GSNO in DMEM and IMDM

Example traces from the NO electrode showing NO release from GSNO (300 μM) in DMEM and IMDM in the absence and presence of SOD (500 $U.ml^{-1}$). Hb (5 μM) abolished the signal in both media. Arrows show addition of compounds as indicated.

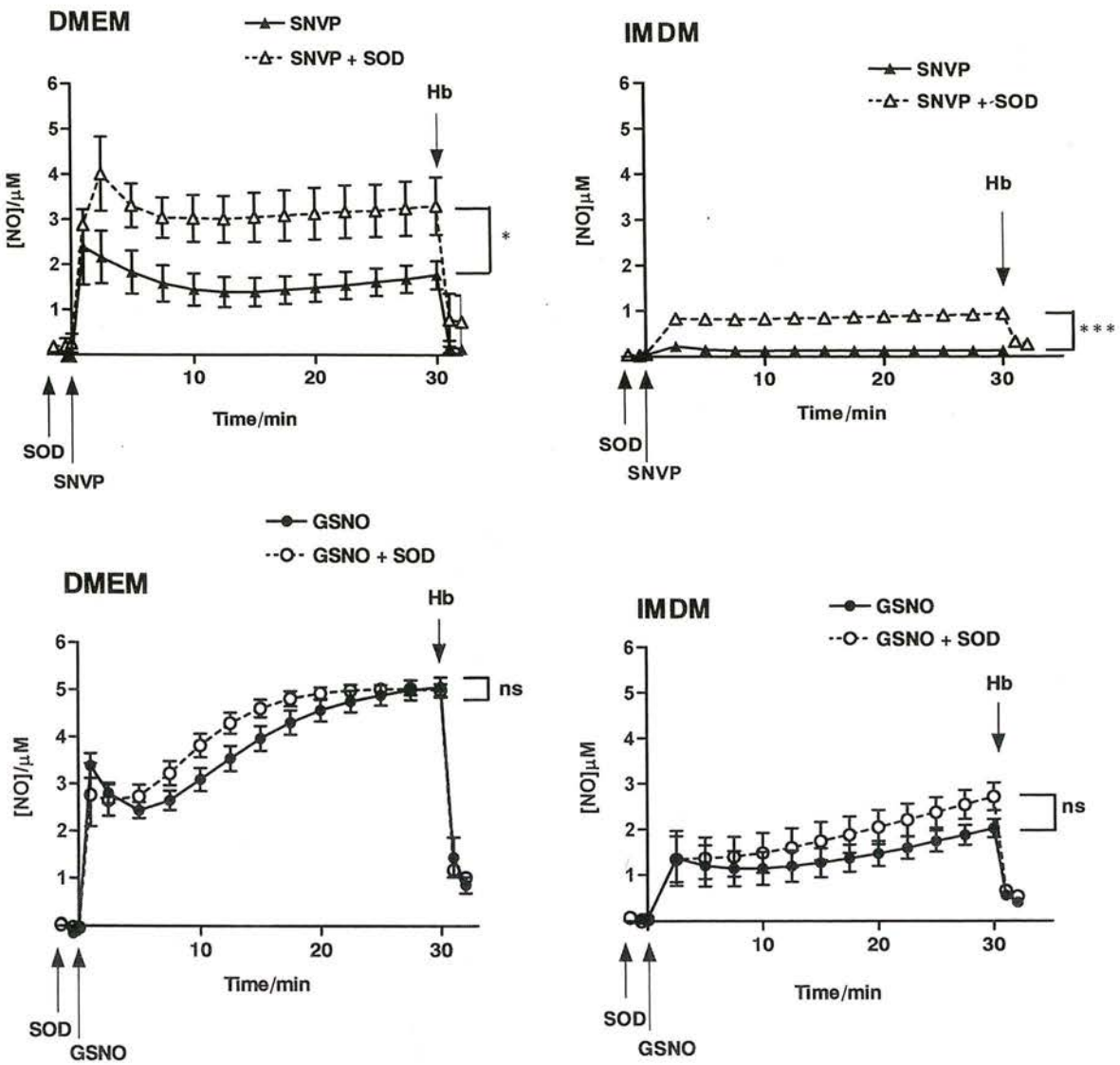

Figure 3.6 NO Release from SNVP and GSNO

NO release from SNVP and GSNO (both 300 μM) was measured by calibrated isolated NO electrode in DMEM (left panel) and IMDM (right panel) in the absence (closed symbols, solid lines) and presence of SOD (500 $U.ml^{-1}$; open symbols, dashed lines). Hb (5 μM) abolished the signal when it had not already decayed to basline. Arrows show addition of compounds as indicated. Analysis of the AUC by unpaired, two-tailed *t*-test revealed SOD significantly increased NO release from SNVP in DMEM (* = $P<0.05$) and IMDM (*** = $P<0.001$; n=6) but did not affect NO release from GSNO in either media (ns = $P>0.05$; n=6 for both compounds in both media).

COMPOUND	DMEM		IMDM		DMEM *vs* IMDM (AUC)
	Peak [NO]/μM	AUC (arbitrary units)	Peak [NO]/μM	AUC (arbitrary units)	
DEA/NO (10 μM)	11.2 ± 2.2	192.0 ± 35.5	8.94 ± 1.0	120.1 ± 16.1	P = ns
DEA/NO (10 μM) + SOD (500 $U.ml^{-1}$)	11.1 ± 1.8	176.9 ± 22.7	10.3 ± 0.6	127.7 ± 15.2	P = ns
DETA/NO (300 μM)	3.9 ± 0.1	97.7 ± 2.8	2.6 ± 0.2	67.1 ± 6.4	P<0.01
DETA/NO (300 μM) + SOD (500 $U.ml^{-1}$)	4.1 ± 0.4	104.0 ± 10.2	2.6 ± 0.2	70.7 ± 5.4	P < 0.05
SNVP (300 μM)	2.4 ± 0.8	42.3 ± 9.2	0.4 ± 0.1	4.3 ± 1.3	P < 0.01
SNVP (300 μM) + SOD (500 $U.ml^{-1}$)	4.0 ± 0.8	85.9 ± 13.1	0.94 ± 0.1	22.9 ± 1.7	P < 0.01
GSNO (300 μM)	5.1 ± 0.2	113.2 ± 5.8	2.0 ± 0.2	40.9 ± 9.3	P < 0.001
GSNO (300 μM) + SOD (500 $U.ml^{-1}$)	5.0 ± 0.1	122.8 ± 4.8	2.7 ± 0.3	53.3 ± 10.8	P < 0.001

Table 3.1 NO Release from NO Donor Compounds in DMEM and IMDM

NO release from each compound in DMEM and IMDM was measured by isolated NO electrode. Table 3.1 shows a summary of the maximum concentration of NO release (μM) together with the area under the curve (AUC; arbitrary units; figures 3.3 and 3.6) for each NO donor compound in the presence and absence of SOD (500 $U.ml^{-1}$). Analysis of the AUC by unpaired, two-tailed *t*-test revealed differences between the NO release from each NO donor compound in DMEM compared to IMDM as shown in the final column (n=6 for all compounds in both media).

Unlike the diazeniumdiolate and the RS-N=O compounds, GEA-3162 (300 μM) failed to release NO in solution except in the presence of SOD (50 – 500 $U.ml^{-1}$; figures 3.7 & 3.8), when significantly more NO was released in DMEM compared to IMDM (NO concentrations in the presence of maximum concentrations of SOD of 2.3 ± 0.2 μM vs 1.6 ± 0.1 μM; $P < 0.001$, unpaired, two-way analysis of variance (ANOVA); n=6; figure 3.8). Hb (5 μM) abolished the signal from GEA-3162 in the presence of the final concentration of SOD (figures 3.7 & 3.8). Additionally, the release of NO from GEA-3162 (300 μM) in the presence of SOD (500 $U.ml^{-1}$) was of shorter duration in DMEM compared to IMDM (figures 3.9). In DMEM, GEA-3162 in the presence of SOD produced a greater initial release of NO with the signal decaying to baseline by approximately 100 min, whereas in IMDM, the initial release of NO from GEA-3162 was blunted in comparison, but more prolonged overall, with the signal persisting until approximately 260 min (figures 3.9).

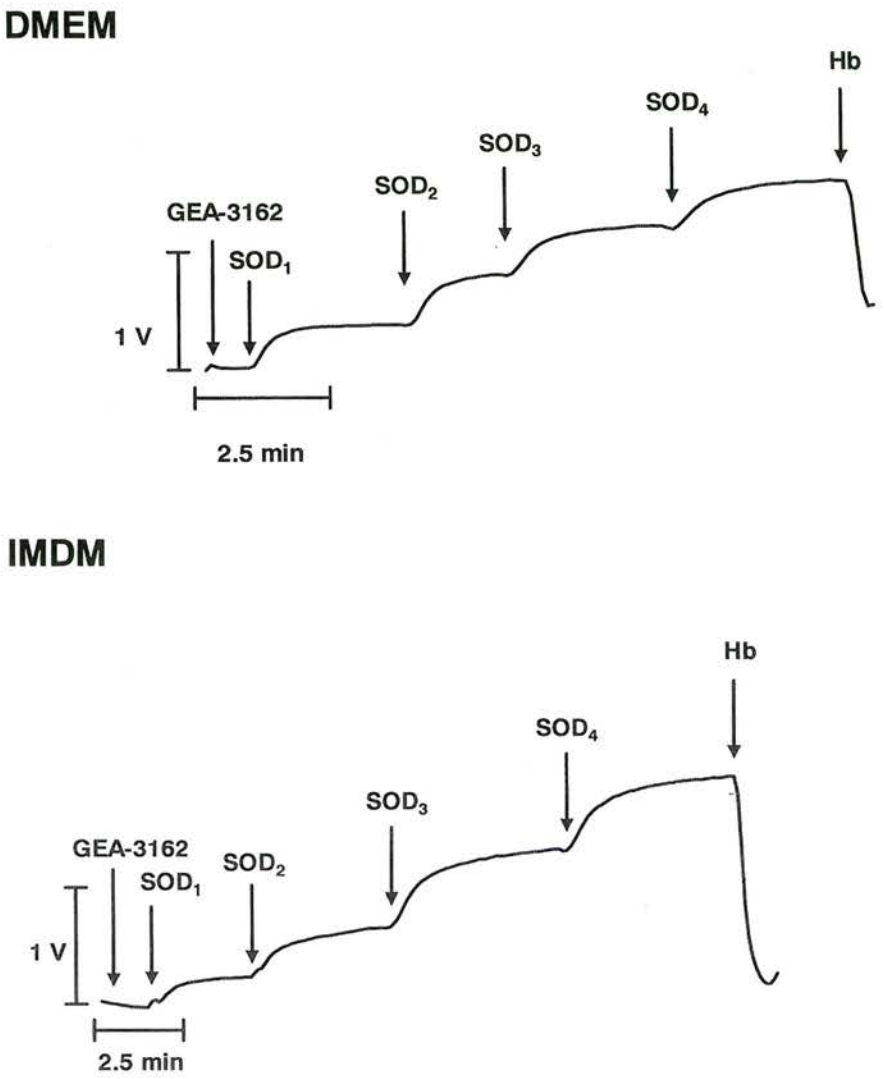

Figure 3.7 NO Release from GEA-3162 in the Presence of SOD in DMEM and IMDM

Example traces from the NO electrode showing NO release from GEA-3162 (300 μM) in the presence of SOD (50 –500 $U.ml^{-1}$) in DMEM and IMDM. Arrows show addition of compounds as indicated. Final SOD concentrations ($U.ml^{-1}$): SOD_1 = 50; SOD_2 = 150; SOD_3 = 300; SOD_4 = 500. Hb (5 μM) abolished the signal in both media when the maximum concentration of SOD had been attained.

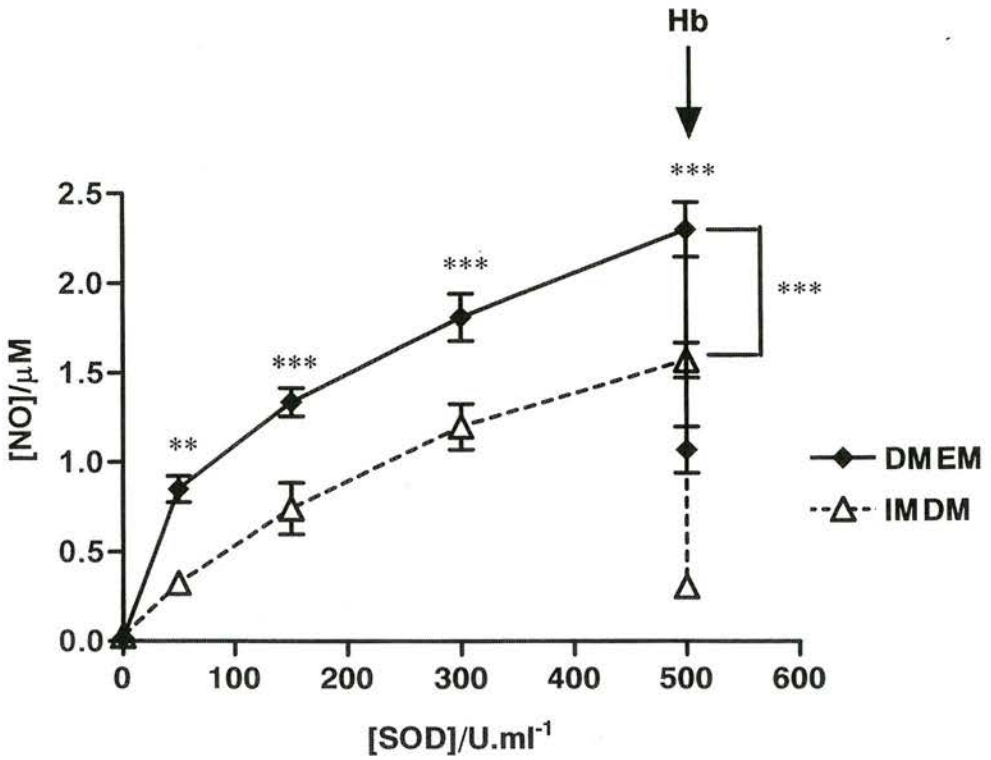

Figure 3.8 NO Release from GEA-3162 in the Presence of SOD

NO release from GEA-3162 (300 μM; A) was measured by calibrated isolated NO electrode in DMEM (solid symbols; solid line) and IMDM (open symbols; dashed line) in the presence of SOD (50 – 500 $U.ml^{-1}$). Arrow indicates addition of the NO scavenger Hb (5 μM). Unpaired, two-way ANOVA followed by post hoc Bonferroni test revealed GEA-3162 (300 μM) released significantly more NO in DMEM compared to IMDM (*** = $P<0.001$; n=6).

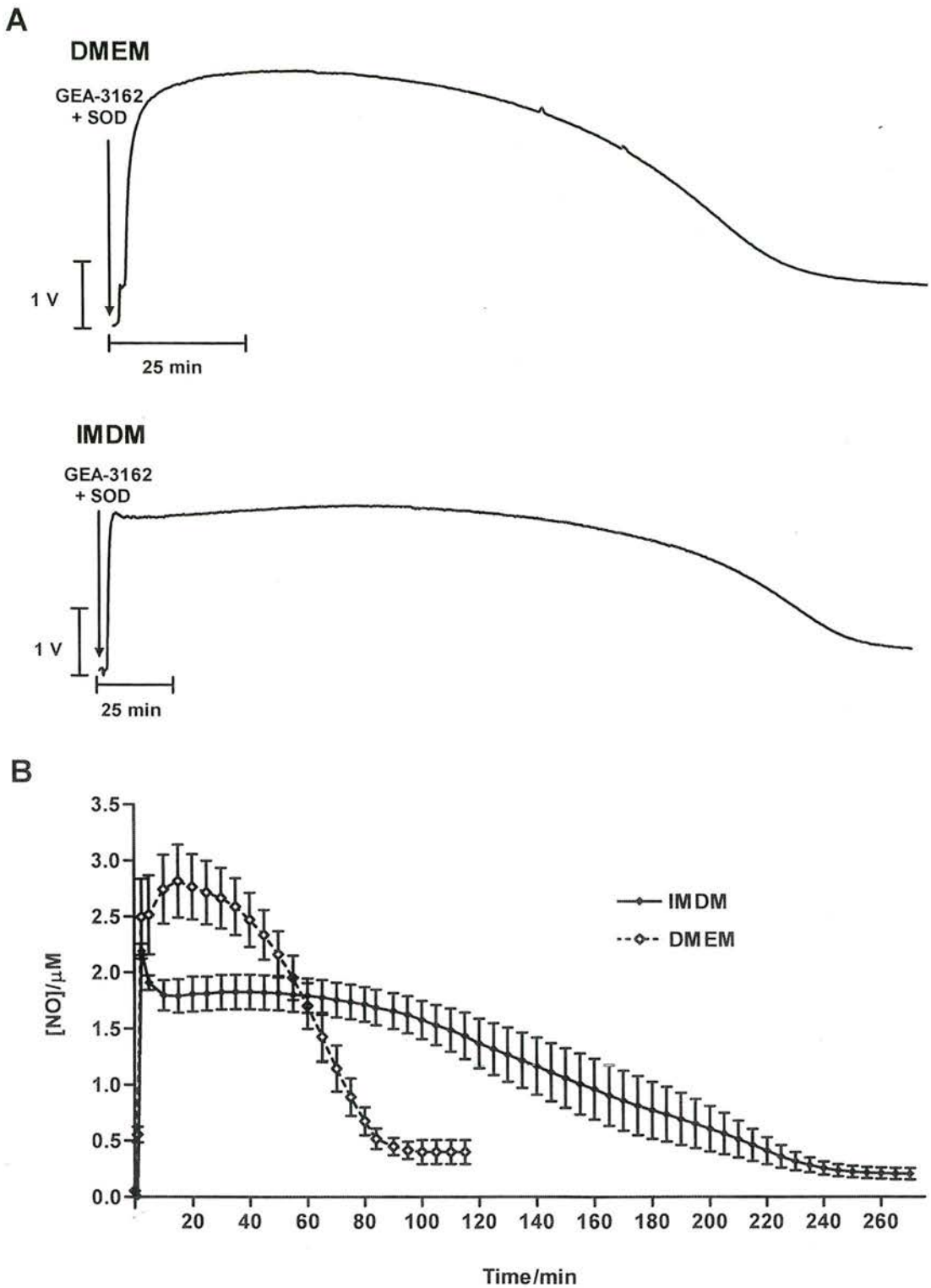

Figure 3.9 Release Profile of NO from GEA-3162 in the Presence of SOD

Example traces from the NO electrode (A) and average graph (B; n=6) showing release profile of NO from GEA-3162 (300 μM) in the presence of SOD (500 U.ml-1) in DMEM and IMDM. GEA-3162 and SOD were introduced simultaneously into the electrode chamber and the signal recorded until it had decayed to baseline. Arrows show addition of compounds as indicated.

In summary, NO release from all the NO donor compounds, with the exception of DEA/NO, varied accorded to the medium in to which they were introduced. In all cases where differences were observed, the measurable NO release was greater, (and shorted lasting in the case of GEA-3162) in DMEM compared to IMDM. In order to investigate if these differences were due to different means of buffering between the two media – $NaHCO_3$ for DMEM and HEPES for IMDM (see appendix one), pilot experiments were conducted to measure NO release from each compound in $NaHCO_3$-buffered H_2O and HEPES-buffered H_2O.

In $NaHCO_3$- buffered H_2O (DMEM equivalent), DEA/NO (5 μM) released a peak NO concentration of 7.4 μM at approximately 5 min, which had not completely decayed to baseline at the end of the 30 min recording period (figure 3.10). In HEPES-buffered H_2O (IMDM equivalent), the peak signal from DEA/NO was marginally greater (8.1 μM), occurred slightly earlier (at 2 min) and had decayed to baseline by ~20 min (figure 3.10).

DETA/NO (300 μM) released a peak concentration of 2.6 μM NO in $NaHCO_3$-buffered H_2O compared to 1.4 μM NO in HEPES-buffered H_2O (figure 3.10). The profile of NO release from DETA/NO was similar in both solutions, reaching a plateau by approximately 10 min and remaining steady throughout the 30 min recording, at the end of which the signal was abolished by Hb (5 μM; figure 3.10).

NO release from SNVP (300 μM) was greater in HEPES-buffered H_2O (IMDM equivalent) than in $NaHCO_3$-buffered H_2O (7.7 μM *vs* 2.4 μM; figure 3.10). Hb abolished the signal from SNVP at the end of the 30 min recording in both solutions (figure 3.10). NO release from GSNO (300 μM) was limited in both

solutions: 0.3 μM in $NaHCO_3$-buffered H_2O and 0.1 μM in HEPES-buffered H_2O (figure 3.10). Hb abolished the signal to baseline at the end of the 30 min recording in both solutions (figure 3.10).

In $NaHCO_3$-buffered H_2O (DMEM equivalent), GEA-3162 (300 μM) in the presence of SOD (500 $U.ml^{-1}$) produced a maximum concentration of 4.9 μM NO by approximately 2.5 min, which remained constant for approximately 80 min before slowly decaying to baseline by approximately 160 min (figure 3.10). In HEPES-buffered H_2O, the peak concentration of NO released from GEA-3162 in the presence of SOD occurred at approximately the same time (2.5 min), but was slightly greater (6 μM) and decayed rapidly during the first 30 min of recording (figure 3.10).

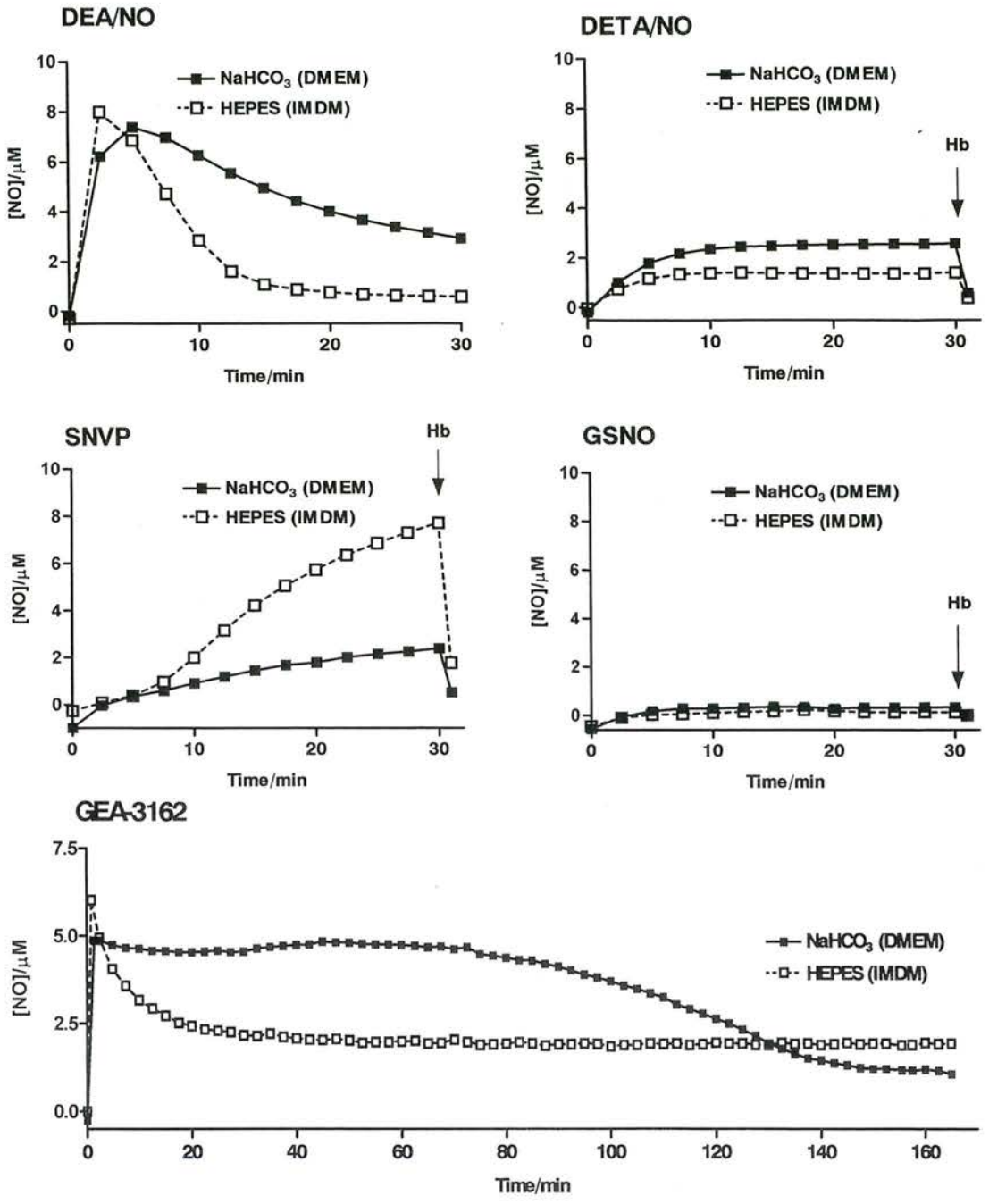

Figure 3.10 NO Release in $NaHCO_3$ and HEPES Buffered H_2O

NO release from DEA/NO (5 μM), DETA/NO, SNVP, GSNO, and GEA-3162 (all 300 μM) was measured by calibrated isolated NO electrode in $NaHCO_3$-buffered H_2O (DMEM equivalent; closed symbols; solid line) and HEPES-buffered H_2O (IMDM equivalent; open symbols; dashed line). Hb (5 μM) abolished the signal from DETA/NO, SNVP and GSNO at the end of the 30 min recording (indicated by arrows). The signal from GEA-3162 was measured in the presence of SOD (500 $U.ml^{-1}$). n=1 for both solutions.

Since the different buffering methods did not fully account for the differences observed in NO release between the two media, the effect of the presence of protein in the media was investigated by repeating the recordings in IMDM plus 10% FCS (as is supplemented to DMEM).

In IMDM containing FCS (10%), DEA/NO (5 μM) released a maximum concentration of 6.5 μM NO, with the peak occurring at approximately 5 min and decaying to baseline by approximately 20 min (figure 3.11). DETA/NO (300 μM) released 10.8 μM NO in IMDM containing FCS, with the signal reaching a plateau by approximately 15 min, and being abolished to baseline by Hb (5μM) at the end if the 30 min recording period (figure 3.11).

SNVP (300 μM) released a maximum NO concentration of 2.8 μM in IMDM containing FCS, with the peak signal occurring at approximately 10 min before slowly decaying; Hb added at the end of the 30 min recording period abolished the signal (figure 3.11). GSNO (300 μM) released a maximum NO concentration of 9.3 μM, with the peak signal occurring at approximately 2.5 min before slowly decaying and being abolished to baseline by Hb at the of the 30 min recording period (figure 3.12.

In IMDM containing FCS, GEA-3162 (300 μM) in the presence of SOD (500 $U.ml^{-1}$) produced a maximum concentration of 7.4 μM NO by approximately 25 min, which slowly decayed to baseline by approximately 260 min (figure 3.11).

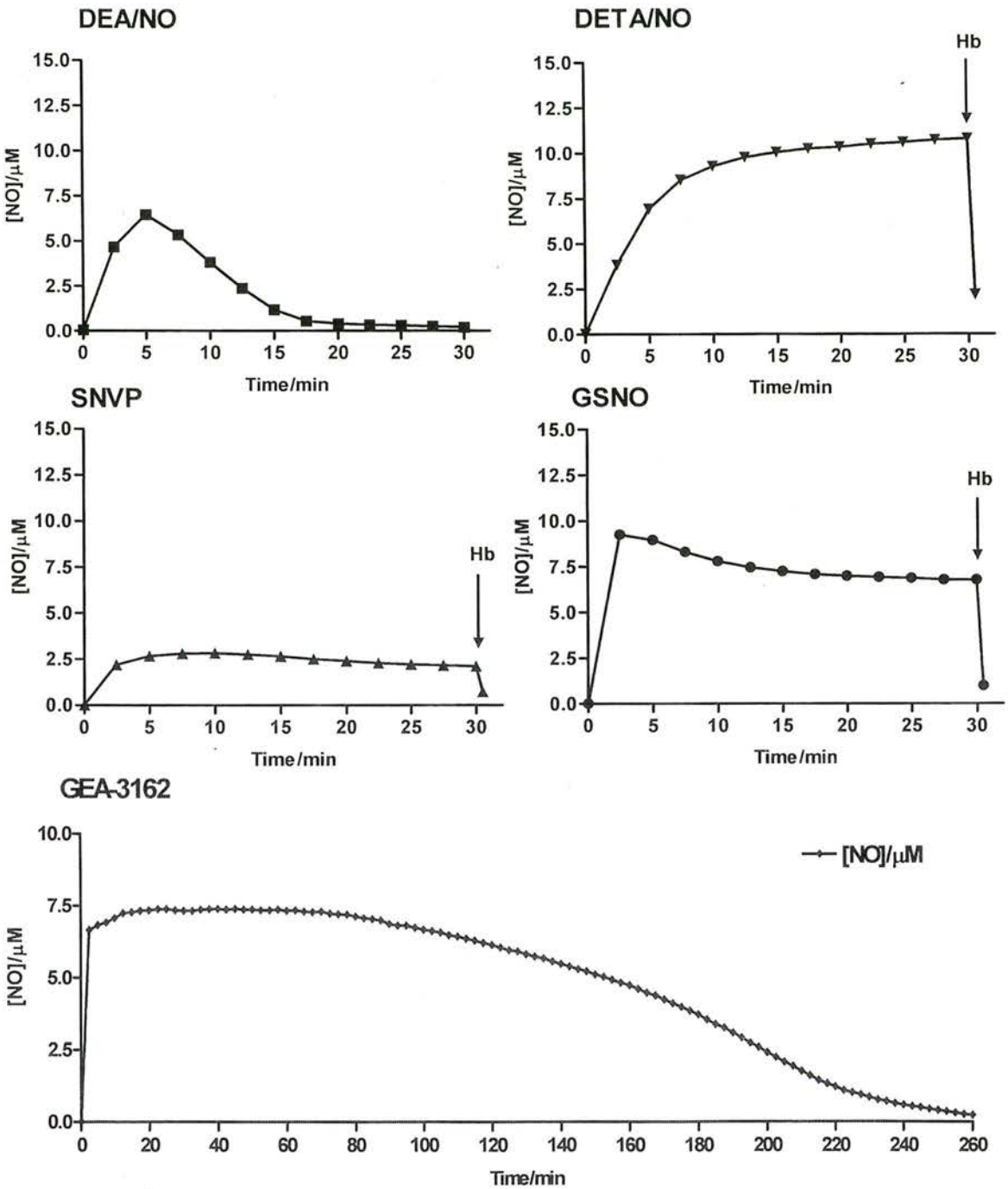

Figure 3.11 NO Release in IMDM Containing 10% FCS

NO release from DEA/NO (5 μM), DETA/NO, SNVP and GSNO (all 300 μM) was measured by calibrated isolated NO electrode in IMDM containing FCS (10%). Hb (5 μM) abolished the signal from DETA/NO, SNVP and GSNO at the end of the 30 min recording (indicated by arrows). The signal from GEA-3162 was measured in the presence of SOD (500 $U.ml^{-1}$). n=1 for all compounds.

3.3.2 EPR Studies

Detection of an EPR signal consistent with the production of the radical adduct, 4-oxo-tempone, was observed only from GEA-3162 (10 – 300 μM) in DMEM and IMDM (figures 3.12 – 3.14). The signal generated by GEA-3162 was significantly attenuated by SOD (500 $U.ml^{-1}$), which typically reduced the intensity of the signal by approximately half in both media (figure 3.12 – 3.14). Levels of oxidising radical species generated by GEA-3162 were significantly greater in DMEM compared to IMDM ($P < 0.001$; unpaired, two-way ANOVA followed by post hoc Bonferroni's test; n=6; figure 3.15).

The diazeniumdiolate compounds, DEA/NO (10 – 100 μM) and DETA/NO (10 – 300 μM), and RS-N=O compounds, SNVP and GSNO (both 10 – 300 μM), failed to generate EPR signals of greater intensity than control and this was unaffected by the presence of SOD (data not shown). The highest DEA/NO concentration (300 μM) did generate a signal significantly greater than control, but SOD failed to abrogate this signal in both media (5700 ± 770 *vs* 6385 ± 880; $P>0.05$ for DMEM and 4730 ± 380 *vs* 4450 ± 430; $P>0.05$ from IMDM; unpaired, two-tailed t-test; n=6 for both media).

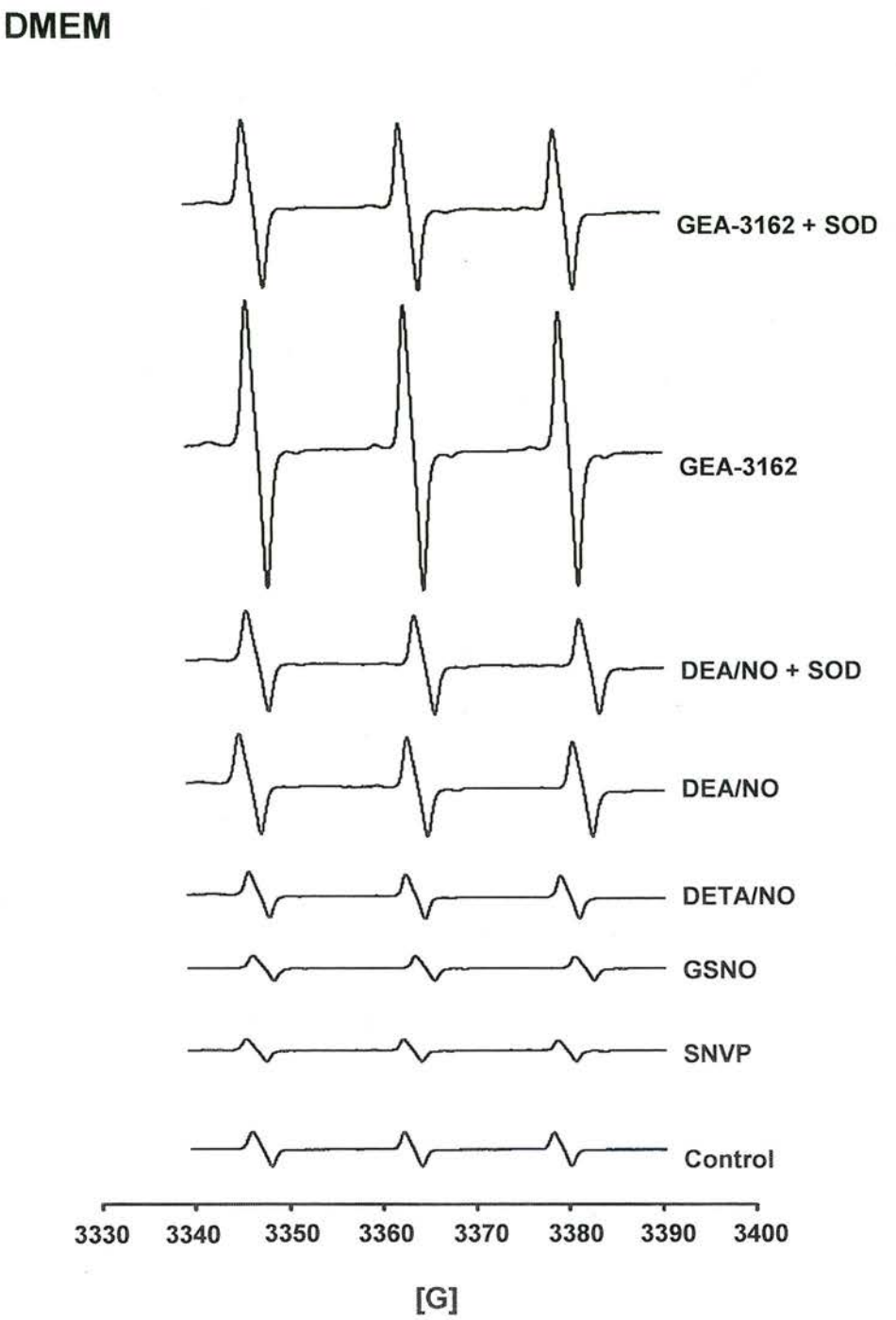

Figure 3.12 Oxidising Radical Production by NO Donor Compounds in DMEM

Example EPR spectra showing the signal produced by the radical adduct, 4-oxo-tempo, formed by reaction of the spin trap, Tempone-H (1 mM) with NO compounds (300 μM) as indicated in the right hand legend. Recordings were performed in DMEM (plus supplements) in the presence and absence of SOD (500 $U.ml^{-1}$) as indicated in legends.

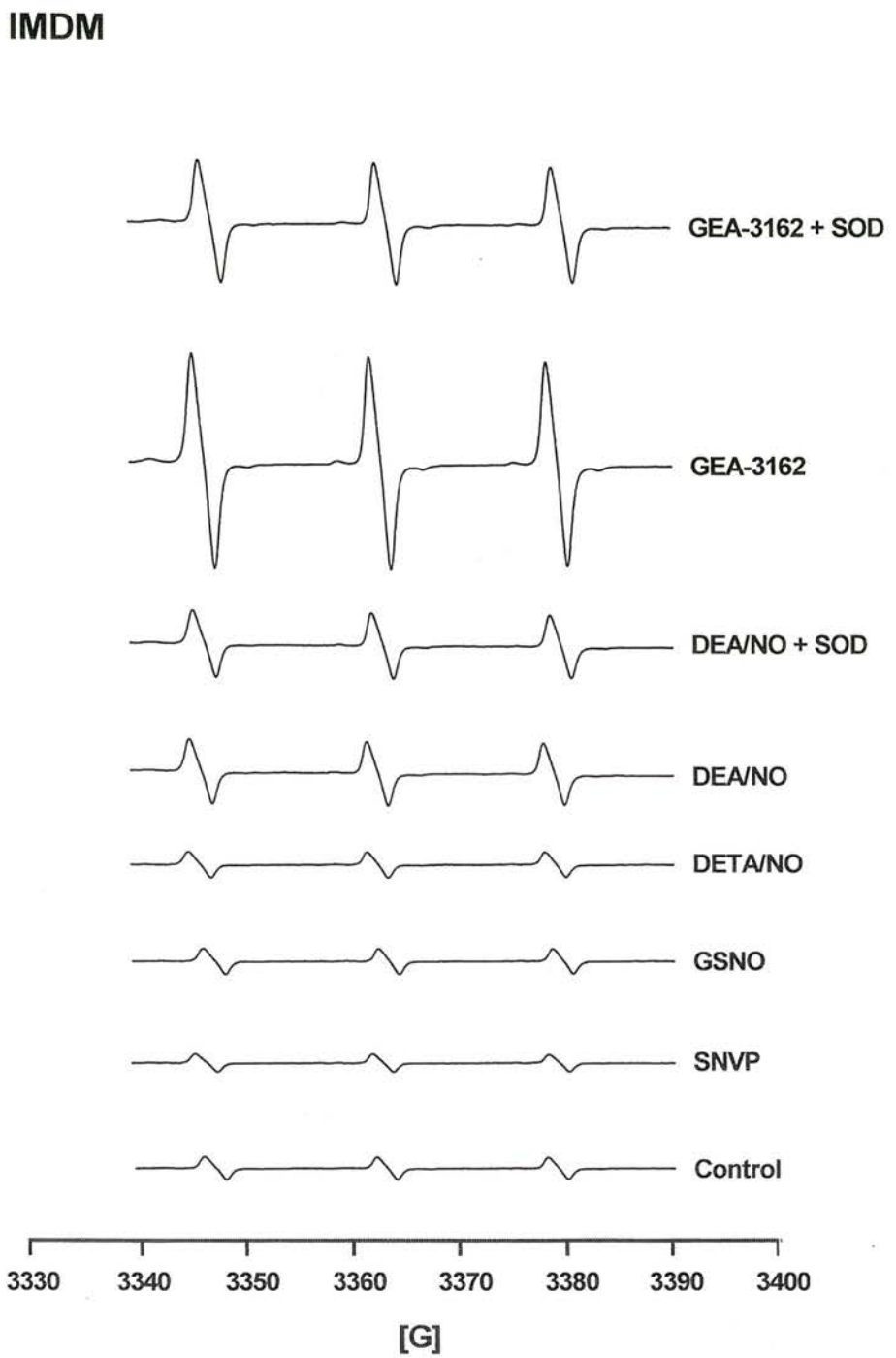

Figure 3.13 Oxidising Radical Production by NO Donor Compounds in IMDM

Example EPR spectra showing the signal produced by the radical adduct, 4-oxo-tempo, formed by the reaction of the spin trap, Tempone-H (1 mM) with NO compounds (300 μM) as indicated in the right hand legend. Recordings were performed in IMDM (plus supplements, but without serum) in the presence and absence of SOD (500 $U.ml^{-1}$) as indicated in legends

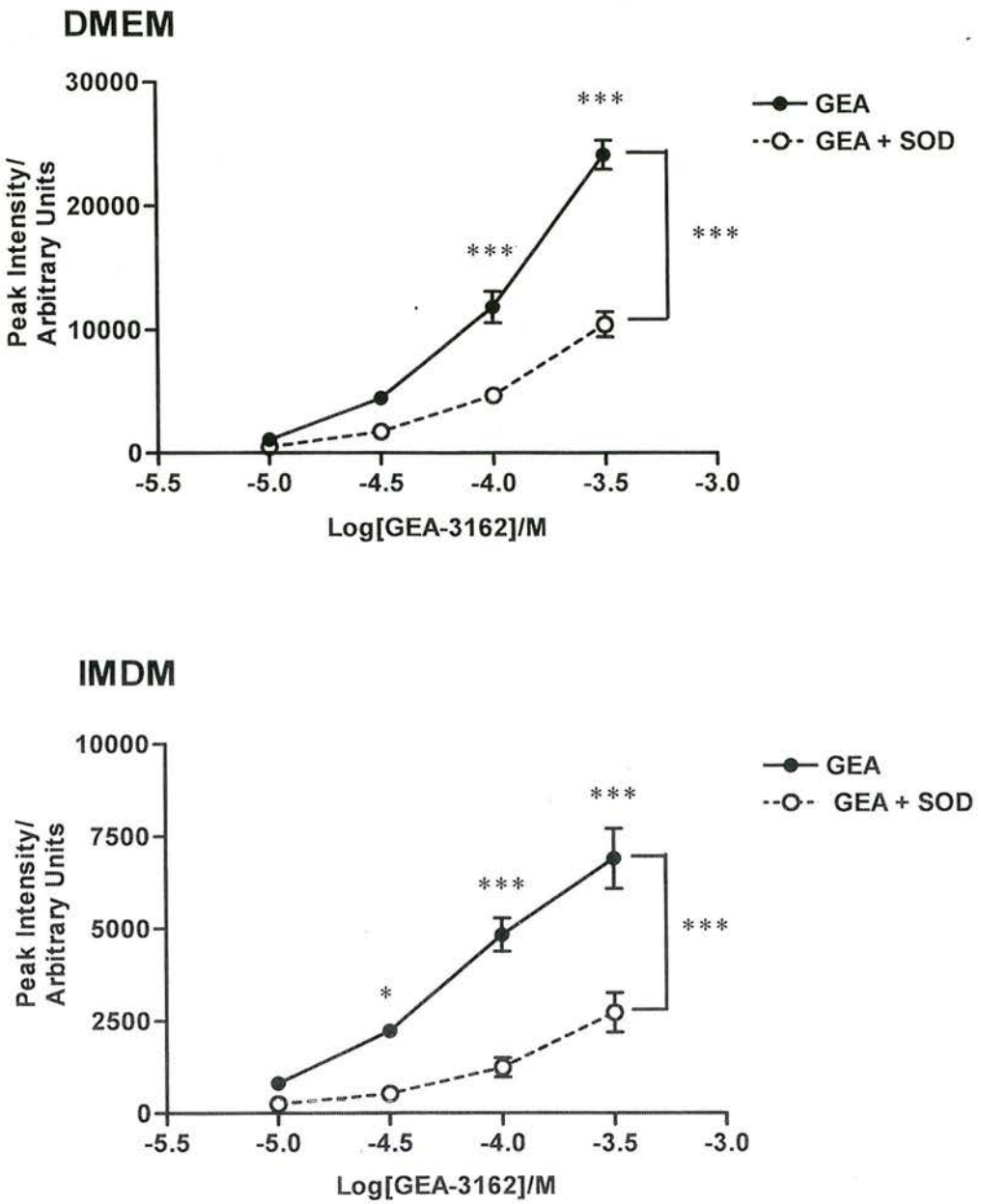

Figure 3.14 Oxidising Radical Production by GEA-3162 in DMEM and IMDM

GEA-3162 (10 – 300 μM; closed symbols, solid lines) generated significant levels of oxidising radical species in DMEM and IMDM. Analysis by unpaired, two-way ANOVA followed by post hoc Bonferroni's test revealed SOD (500 $U.ml^{-1}$; open symbols, dashed lines) significantly attenuated the signal from GEA-3162 (* = $P<0.05$, ** = $P<0.001$; *** = $P<0.001$; n=6 for both media).

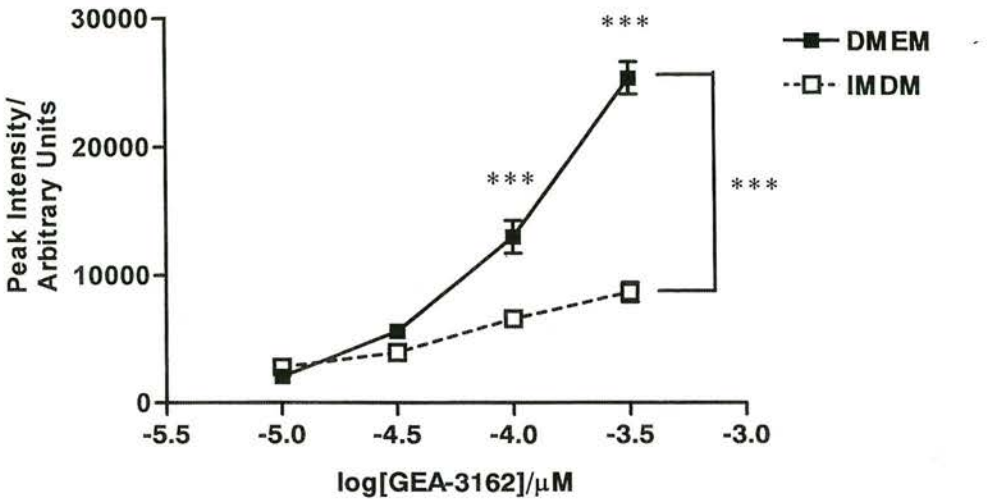

Figure 3.15 Oxidsing Radical Production by GEA-3162 in DMEM and IMDM

Levels of oxidising radical species generated by GEA-3162 (10 – 300 μM) were significantly greater in DMEM (closed symbols, solid line) compared to IMDM (open symbols, dashed lines). *** = $P < 0.001$, unpaired, two-way ANOVA followed by post hoc Bonferrroni test; n = 6 for both media)

3.4 Discussion

These studies provide characterisation of the NO-related species liberated by three different classes of putative NO donor compound. Variations in the concentration and the release profile of NO and NO-related species in solutions of varying composition justified the necessity to conduct such characterisation studies in conditions closely reflecting those employed in subsequent biological studies.

Studies using the isolated NO electrode demonstrated that the diazeniumdiolates spontaneously liberate NO radical in solution, with release

profiles consistent with their half-lives and unaffected by the presence of SOD. In common with previously published data on this class of compound (Fitzhugh and Keefer 2000; Davies et al. 2001; Arnold et al. 2002), both DEA/NO and DETA/NO released NO radical as would be predicted from their published half-lives, i.e. a rapid and short burst of NO from DEA/NO ($t_{½}$=2 min), and slower, more prolonged delivery of NO from DETA/NO ($t_{½}$=20 h). The NO-scavenger, Hb, verified that the signal recorded from DETA/NO was indeed due to NO. These observations are unsurprising because members of this class of compound are known to spontaneously liberate NO in solution without the need for enzymatic activation, or the presence of, for example, tissue thiol groups (Megson 2000; Megson and Webb 2002).

Unlike the diazeniumdiolate compounds, RS-N=Os liberate only very low levels of NO radical spontaneously in solution, with the peak NO concentration generated being several hundred fold lower, and bearing an unpredictable relationship, to the concentration of the RS-N=O compound. The small NO electrode signals generated by SNVP and GSNO were confirmed to be due to NO by the successful abolition of the signal by Hb.

Interestingly, both SNVP and GSNO generated a biphasic release profile of NO with rapid initial spike, followed by a slower release of NO. This could result from the combination of two separate phases of NO generation as illustrated in figure 3.16.

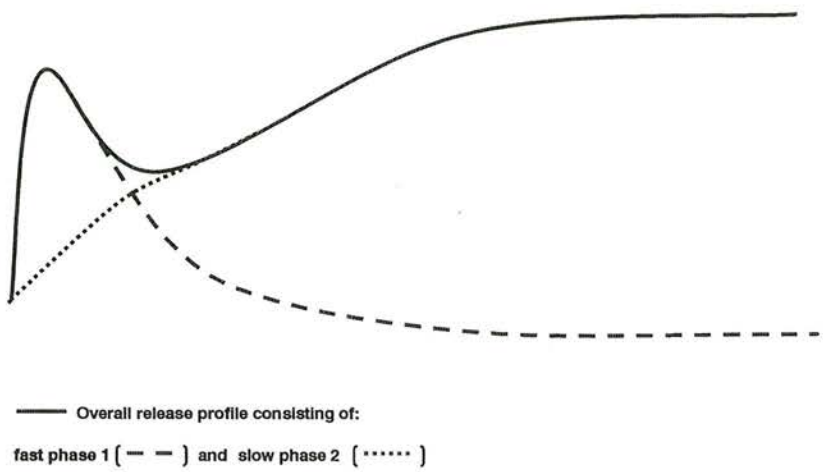

Figure 3.16 Schematic Representation of NO Release from RS-N=O

NO release from RS-N=O compounds occurred in a biphasic manner which may resulted from a composite of two individual phases: an initial fast phase and a subsequent slow phase as indicated.

The mechanism of RS-N=O decomposition remains to be fully elucidated but there are some interesting theories that could explain this biphasic phenomenon. Firstly, decomposition of RS-N=O compounds is greatly accelerated in the presence of Cu^{+}, which catalyses the breakdown of RS-N=O to yield NO plus the corresponding disulphide group (RS-SR) (Gordge et al. 1995; Dicks and Williams 1996; Gorren et al. 1996; Singh et al. 1996; Al-Sa'doni et al. 1997; Butler and Rhodes 1997). Exceedingly low concentrations of trace metal ions, including those present in distilled laboratory water and most buffers, are capable of accelerating RS-N=O decomposition (Butler et al. 1995). Additionally, reduction of Cu^{2+} bound in proteins has been proposed as a mechanism to release NO from RS-N=O *in vivo* (Dicks and Williams 1996). Acceleration of decomposition due to protein-bound

Cu^{2+} might explain why the level of NO generated by both GSNO and SNVP in DMEM, which is supplement with bovine foetal calf serum (FCS; 10%) as a protein source, is greater than that generated in IMDM, which during NO electrode studies, was protein-free. Indeed, pilot experiments in which IMDM was supplement with 10% FCS, resulted in an increase in the level of NO generated. However, the presence of protein may also influence NO release from RS-N=O in additional ways. For example, transnitrosation reactions, involving rapid transfer of NO^+ from the RS-N=O to a reduced cyteine group of a protein, may yield a simpler S-nitrosothiol that is more susceptible to Cu^+-induced decomposition (Park 1988; Kowaluk and Fung 1990; Askew et al. 1995; Liu et al. 1998; Hogg 1999; Ceron et al. 2001).

Secondly, RS-N=O may undergo enzymatic cleavage of a peptide bond to yield the corresponding disulphide and an S-nitrosothiol that is more susceptible to Cu^+-induced decomposition (Askew et al. 1995). GSNO has been demonstrated to be a substrate for the enzyme γ- glutamyltranspeptidase (γ-GT) (Askew et al. 1995; Hogg et al. 1997), which is normally involved in the metabolism of glutathione (Tate and Meister 1981; Keillor et al. 2005). Following catalytic breakdown by γ-GT, GSNO yields glutamic acid and the S-nitrosothiol, S-nitrosoglycylcysteine, from which NO can be subsequently liberated by metal ion catalysis (Askew et al. 1995; Hogg et al. 1997).

Finally, it has been demonstrated that decomposition of GSNO may not go to completion, especially at high concentrations, due to the ability of the resulting disulphide group (GS-SG) to act as a metal ion chelator, hence removing, or slowing, metal ion-induced catalysis (Swift 1996; Noble and Williams 2000).

Considered in combination, metal ion catalysis followed by chelation of metal ions by the breakdown products, and transnitrosation and/or enzymatic cleavage of a peptide bond, prior to subsequent metal ion catalysis, may account for the biphasic release profile of NO from GSNO and SNVP presented in the current study. In this model, metal ion catalysis could potentially induce the initial fast phase, which then rapidly declines due to chelation of metal ions by the resulting disulphide product. Transnitrosation or enzymatic cleavage of a peptide bond prior to subsequent metal ion induced catalysis is then responsible for the second slower phase of NO release. This phase proceeds despite chelation of metal ions by the disulphide groups formed during the initial phase, because the simpler S-nitrosothiols resulting from the transnitrosation or enzymatic cleavage are more susceptible to metal ion catalysis, therefore, their decomposition can be catalysed by a lower concentration of ions.

However, it is unclear whether the disulphide product resulting from SNVP decomposition would be capable of chelating Cu^{+} or whether SNVP would be a suitable substrate for γ-GT. This may explain why the biphasic profile of NO release is more pronounced for GSNO than SNVP. Additionally, no direct source of γ-GT, or similar peptidase enzymes have been identified in the reagents used in these experiments. It is possible that such enzymes may be present in bovine FCS, accounting for the more marked biphasic effect in DMEM that IMDM. However, the FCS is heat inactivated (1 hour; 50°C; chapter two, section 2.2.3), which is likely to have denatured any enzymes present. Furthermore, although addition of FCS to IMDM in a pilot study did enhance the biphasic profile of NO release from GSNO, it remained less pronounced than in DMEM and FCS failed to elicit a marked change

in the release profile of SNVP. For these reasons, transnitrosation is possibly more likely than enzymatic cleavage under the experimental conditions used in the current study.

Metal ion catalysis is also likely to account for the observed increase in NO release from SNVP in the presence of SOD. Given that low concentrations of Cu^{+} greatly accelerate the decomposition of RS-N=O, copper present in the Cu/Zn SOD isoform used in these studies is likely to be sufficient to augment NO release from RS-N=O compounds. Although SOD tended to increase NO release from GSNO, this trend did not reach significance, possibly due to metal ion chelation by the disulphide production of GSNO decomposition, GS-SG (Swift 1996; Noble and Williams 2000), which has previously been demonstrated to limit SOD-dependent decomposition of GSNO (Singh et al. 1999).

GEA-3162 failed to liberate NO in solution except in the presence of SOD. SOD converts O_2^- to H_2O_2, thus preventing O_2^- and NO combining to form $ONOO^-$ (Beckman and Koppenol 1996). Although the reaction of NO and O_2^- is diffusion-limited ($k>10^9$ $M^{-1}S^{-1}$), (Huie and Padmaja 1993; Espey et al. 2000; Wolin et al. 2002), SOD is present at high concentrations in these experiments and likely to be in excess. This allowed SOD to successfully compete with NO for O_2^-, thus preventing the formation of $ONOO^-$, and effectively 'unmasking' the NO generated by GEA-3162. This 'unmasked' signal generated by GEA-3162 in the presence of SOD was confirmed to be a genuine NO signal by the successful abolition of the unmasked signal by the NO-scavenger, Hb. The duration of the NO signal in the presence of SOD was in the region of several hours, therefore, it is reasonable to infer that GEA-3162 decomposes over a period of several hours.

These initial electrode studies suggested that GEA-3162 was generating NO and O_2^- concomitantly. This was further illustrated by the EPR studies demonstrating significant GEA-3162-induced oxidation of Tempone-H, forming the radical adduct, 4-oxo-tempone. Tempone-H has successfully been used previously as a method for the detection of O_2^- and $ONOO^-$ (Dikalov et al. 1997a; Dikalov et al. 1997b). There are two possibilities as to how GEA-3162 might bring about the oxidation of Tempone-H to result in formation of 4-oxo-tempone and the consequent EPR signal. Firstly, Tempone-H could be directly reacting with $ONOO^-$ in a two-step process involving the production of NO_2 as an intermediary, with the result that one molecule of $ONOO^-$ finally forms two molecules of 4-oxo-tempone (reactions 1 and 2) (Dikalov et al. 1997a).

$$\text{Tempone-H} + ONOO^- \longrightarrow \text{4-oxo-tempone} + \bullet NO_2 + H_2O \quad (1)$$

$$\text{Tempone-H} + \bullet NO_2 \longrightarrow \text{4-oxo-tempone} + HNO_2 \quad (2)$$

Alternatively, if GEA-3162 releases NO and O_2^- concomitantly, then Tempone-H could be competing with NO for O_2^-, resulting in the oxidation of Tempone-H by O_2^- (reaction 3), before NO and O_2^- combine to form $ONOO^-$.

$$\text{Tempone-H} + O_2^- \longrightarrow \text{4-oxo-tempone} + H_2O_2 \quad (3)$$

Dikalov *et al* have calculated the overall rate constant for reaction 1 + reaction 2 to be $k_{1+2} = 6 \times 10^9\ M^{-1}s^{-1}$ and for reaction 3 to be $k_3 = 1.2 \times 10^4\ M^{-1}s^{-1}$ (Dikalov et al. 1997a), whilst the rate constant for the reaction of NO with O_2^- is in

the region of $k > 10^9$ $M^{-1}s^{-1}$ (Huie and Padmaja 1993; Wolin et al. 2002). Given a consistent Tempone-H concentration and the diffusion-limited rate constant of the reaction of NO with O_2^-, one might predict that reactions 1 + 2 are the more likely to occur and that therefore, Tempone-H is oxidised by $ONOO^-$ rather than O_2^-. SOD is able to attenuate the EPR signal generated by GEA-3162 because this antioxidant enzyme combines with O_2^- at a rate comparable with that of NO (Espey et al. 2000), and is present at high enough concentrations to successfully compete with NO for O_2^-. Therefore, the O_2^- generated is scavenged by SOD, thus preventing the formation of $ONOO^-$, and hence reducing the concentration of $ONOO^-$ available to oxidise Tempone-H.

Taken together, these two lines of evidence demonstrate that GEA-3162 decomposes to co-generate NO and O_2^- concomitantly and should therefore, be regarded as a $ONOO^-$ generator, rather than an NO donor. These observations confirm earlier studies demonstrating similar results for both GEA-3162 and the structurally similar compound, SIN-1 (Taylor et al. 2004). Although initial studies demonstrated that GEA-3162, unlike SIN-1, was able to liberate NO in the absence of O_2^- generation (Kankaanranta et al. 1996; Holm et al. 1998), these earlier studies used indirect methods to measure both NO and O_2^-, rather then more direct and accurate methods presented in the current study.

Somewhat surprisingly, at high concentrations, DEA/NO generated a significant EPR signal, appearing to suggest the production of oxidising radical species from this compound. However, SOD failed to attenuate the EPR signal, therefore it seems likely that the signal generated by high concentrations of DEA/NO indicates loss of specificity of the chemical spin-trap at high concentrations of any

radical, rather than a genuine production of O_2^- or $ONOO^-$. This does not occur at high concentrations of DETA/NO because, as the NO electrode studies demonstrate, the release of NO radical from DETA/NO is far slower than from DEA/NO, and therefore unlikely to reach sufficient concentrations quickly enough to result in loss of specificity of the spin-trap. Neither SNVP nor GSNO generated oxidising radical species in either media. It would not be expected for this class of compound to generate oxidising radicals, and there are no previous reports of them doing so. Indeed, generation of free thiol groups during the decomposition RS-N=O compounds might result in anti-oxidant properties, giving this group of compounds additional anti-atherosclerotic benefits over and above NO generation.

Interestingly, the composition of the solution into which these putative NO donor compounds were introduced had a significant impact on the concentration and release profile of NO. On the whole, DMEM accelerated NO release (or oxidising radical generation in the case of GEA-3162) compared to IMDM. With the exception of DEA/NO, which displayed consistent release characteristics, NO generation was sensitive to the composition of the medium – even the concentration of NO released from DETA/NO, often described as releasing NO in a highly predictable manner (Davies et al. 2001), was influenced by medium composition. Pilot experiments designed to elucidate the components of the medium responsible for this phenomenon demonstrated even something as basic as the method of buffering a solution can influence NO release. DMEM is buffered with sodium bicarbonate ($NaHCO_3$; 44.05 mM) and IMDM with HEPES (25.03 mM; appendix one) but the pH of the media was identical (pH 7.3), therefore, the actual chemical entities appear to alter the decomposition. HEPES has previously been demonstrated

to blunt NO release from diazeniudiolates as a result of the formation of O_2^- during HEPES oxidation (Keynes et al. 2003). However, given that SOD did not affect NO release from DETA/NO in IMDM, and that NO release from DETA/NO was increased in HEPES-buffered H_2O compared to IMDM, this is perhaps not the principle factor responsible for the differences observed between the two media. Although, further pilot studies indicated that the presence of protein (FSC) may be responsible for some of the differences observed between DMEM and IMDM during the decomposition of the RS-N=O, neither the buffering method nor the presence of protein fully rationalised the different pattern of NO release between the media.

Overall, these studies provide characterisation of the NO species liberated by three different class of putative NO donor drug. The results from studies involving the diazeniudiolate compounds support the notion that these compounds are pure NO donors whose half-lives can be used to accurately predict NO release. The precise mechanism of RS-N=O decomposition has not been identified, but the low levels of NO radical and lack of EPR signals generated by this group of compounds confirm that the main mechanism of action or RS-N=O is not via the release of NO radical or oxidising radical species, but by another mechanism that likely involves the transfer of NO^+ and the generation of intermediate S-nitrosothiol species. Importantly, these studies provide strong evidence that GEA-3162 decomposes to co-generate NO and O_2^- and so should be regarded as a $ONOO^-$ generator, rather than an NO donor. Finally, the somewhat surprising observation that NO release is sensitive to the composition of the medium underlines the absolute requirement to conduct this type of preliminary characterisation study. Furthermore, in order to be confident of the

accuracy of this type of data, it is essential to conduct such studies under conditions closely paralleling those employed in subsequent biological investigations.

Chapter Four

The Role of NO-Related Species in Vascular Smooth Muscle Cell Proliferation and Viability

4. The Role of NO and NO-Related Species in Vascular Smooth Muscle Cell Proliferation and Viability

4.1 Introduction

Vascular smooth muscle cells (VSMCs) play an important role in the vascular remodelling that occurs during the response to vessel injury in atherosclerosis. VSMCs constitute the major cellular component of the fibrous cap that forms over the developing atherosclerotic lesion, encapsulating the plaque and serving as a barrier to separate the highly thrombogenic contents of the plaque core from the circulation, thus maintaining the stability of the plaque (Ross 1993; Davies 1997). Plaque instability and rupture are now considered to be the major determinant of the acute clinical consequences of atherosclerosis such as unstable angina, myocardial infarction, and stroke (Davies 1995; Schroeder and Falk 1995; Dalager-Pedersen et al. 1998; Gutstein and Fuster 1999; Zhou et al. 1999; Corti and Badimon 2002; Mitra et al. 2004). Plaque rupture occurs if the cap is disrupted, causing exposure of the contents of the core to the circulation, and resulting in thrombosis. Although the underlying causes of plaque rupture are yet to be fully identified, such events are associated with a reduction in the thickness, and VSMC content, of the plaque cap (Bauriedel et al. 1999; Leskinen et al. 2003). For example, those plaques with a thin fibrous cap containing a relatively low density of VSMCs are considered to have a greater propensity to rupture than those with a thicker cap containing a higher density of VSMC (Davies 1996; Felton et al. 1997). Therefore, in order to maintain the

stability of the plaque, it is essential to preserve the integrity of the VSMC cap by maintaining the population of VSMC within it. Processes inducing cell death, such as apoptosis or necrosis, will reduce the VSMC content of the cap and may have serious consequences for plaque stability by promoting rupture (Kockx and Herman 1998; Bauriedel et al. 1999). Indeed this has been demonstrated in the *ApoE*$^{-/-}$ null murine model of atherosclerosis, where direct induction of VSMC apoptosis induces both rupture and thrombosis of plaques (von der Thusen et al. 2002).

The net balance between proliferation and cell death will determine the VSMC content within the cap. NO can influence both of these processes, exerting an inhibitory effect on proliferation (Garg and Hassid 1989; Kariya et al. 1989; Nakaki et al. 1990; Assender et al. 1992; Newby et al. 1992; Garg and Hassid 1993; Mooradian et al. 1995; Yu et al. 1997; Jeremy et al. 1999) and inducing cytotoxic effects (Fukuo et al. 1995; Nakahashi et al. 1995), including apoptosis (Fukuo et al. 1996; Nishio et al. 1996; Pollman et al. 1996; Nicotera et al. 1997; Bennett 1999; Lau 2003). The anti-proliferative actions of NO are largely considered to be brought about by mechanisms involving cGMP signalling (Kariya et al. 1989; Nakaki et al. 1990; Cornwell et al. 1994; Yu et al. 1997), although a more recent report has suggested a possible cGMP-independent pathway (Ignarro et al. 2001). The mechanism of NO-induced cytotoxicity is less well defined, and in the past has generally been considered to be a result of non-specific oxidative chemistry. However, it is now emerging that this may not be the case, particularly in NO-induced apoptosis where evidence is emerging to establish a more precise role for NO, and active mechanisms that are generally cGMP-independent. For example, NO may act to sensitise cells to apoptotic stimuli by promoting the up-regulation of cell

surface receptors such as tumour necrosis factor receptor 1 (TNF-R1 – the receptor for TNF-α; Boyle et al. 2003), or Fas (the receptor for Fas-L (Boyle et al. 2003).

The precise NO-related species responsible for these anti-proliferative and cytotoxic/pro-apoptotic actions is not entirely clear in VSMCs. Various NO donors have been used as tools to investigate the actions of NO in VSMC proliferation and apoptosis, but most of the studies quoted above have failed to characterise either the concentration of NO, or the NO-related species liberated by such compounds. Some studies have demonstrated that NO-related species, as well as NO *per se*, can induce these responses. For example, $ONOO^-$ has been shown to induce apoptosis in VSMC (Li et al. 2003; Li et al. 2004) and recent studies have demonstrated a central role for signalling by RS-N=O during NO-mediated induction of apoptosis (Foster et al. 2003; Benhar and Stamler 2005; Hara et al. 2005). Due to the paradoxical effects of NO in many settings, investigating NO-species variations in these processes will lead not only to a greater understanding of the role of NO in such processes, but may also offer the opportunity of selective targeting of either a particular cell type, or a particular pathway.

Therefore, the aims of these studies were to investigate the effects of NO, RS-N=O and $ONOO^-$ on the proliferation and viability of VSMC, and to characterise the role of apoptosis or necrosis in any induced cell death.

4.2 Methods

4.2.1 Cell Culture

Bovine aortic smooth muscle cells (BAoSMC) were cultured as described in chapter two (see section 2.2.3).

4.2.2 Detection of NO_2^-/NO_3^- by Griess Test

In order to measure NO_2^-/NO_3^- (NOx) generated from SNVP, GSNO and GEA-3162 (all 10 – 300 μM) in the presence of cells, BAoSMC were seeded in 48-well, flat-bottomed, tissue culture plates and cultured for 24 h (37°C; 95% O_2; 5% CO_2; section 2.2.3) in DMEM. Following this initial 24 h period, the DMEM was aspirated from the wells and replaced with phenol red free DMEM (containing the same supplements as described in section 2.2.3) plus each NO donor compound (10 – 300 μM). This was necessary because the phenol red in DMEM gives the medium a colour that absorbs light at the same wavelength as the Greiss Test, whereas phenol red-free DMEM is colourless.

The total NOx concentration released by SNVP, GSNO and GEA-3162 in tissue culture media in presence of BAoSMC was measured at 1 h, 6 h, and 24 h time points by Griess Test (R&D Systems, Abington, U.K) as described in chapter two (section 2.1.2).

4.2.3 Assessment of Cell Proliferation

Cell proliferation induced by DEA/NO, DETA/NO, SNVP, GSNO and GEA-3162 (all 10 – 300 μM) in BAoSMC was assessed by bromodeoxyuridine (BrdU) assay (Calbiochem, (Merck Biosciences), Darmstadt, Germany) as described in chapter two (section 2.4). The intensity of the coloured product generated is directly proportional to the amount of incorporated BrdU present in the cells.

Cells were incubated (37°C: 95% O_2; 5% CO_2) for 24 h in the presence of BrdU, together with each NO donor compound (10 – 300 μM), or the maximum concentration of drug vehicle (DMSO; 1%: control). Additionally, the experiments were repeated in the presence of the O_2^- scavenger, SOD (500 $U.ml^{-1}$), the specific sGC inhibitor, ODQ (20 μM), or the NO-specific scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.potassium salt (carboxy-PTIO; 500 μM). Results are expressed as absorbance units when comparing NO donor treated cells to control, untreated cells. Alternatively, when comparing NO donor treatments in the presence or absence of SOD, ODQ, or carboxy-PTIO, the results are expressed as a percentage of the appropriate control in order to take into account the absorbance of the inhibitor or scavenger itself.

4.2.4 Assessment of Cell Viability

4.2.4.1 Lactate Dehydrogenase Assay

The cytotoxic impact of DEA/NO, DETA/NO, SNVP, GSNO and GEA-3162, (all 10 – 300 μM) on BAoSMC, was assessed by lactate dehydrogenase (LDH) assay as described in chapter two (section 2.3.2). Additionally, the experiments were

repeated in the presence of SOD (500 $U.ml^{-1}$), ODQ (20 μM) or CarboxyPTIO (500 μM).

4.2.4.2 Flow Cytometry

Cell death induced by NO donor compounds (DEA/NO, DETA/NO, SNVP, GSNO and GEA-3162; all 10 – 300 μM) was characterised as apoptosis or necrosis by flow cytometry as described in chapter two (section 2.3.3).

4.3 Results

4.3.1 NO_2^-/NO_3^- Measurements

The concentration of total NOx generated in the presence of BAoSMC was similar for SNVP, GSNO and GEA-3162, although at higher concentrations total NOx generated by GSNO and GEA-3162 tended to be greater than that produced by SNVP. Statistical analysis (unpaired, one-way ANOVA followed by post hoc Tukey test) revealed a high degree of variability and no overall clear pattern of significant differences between compounds (analyses not shown). The proportion of total NOx consisting of nitrate (NO_3^-) was exceedingly low and approximately equal to that present in DMEM, therefore, the vast majority of NOx generated by the NO donor compounds was nitrite (NO_2^-; figures 4.1 & 4.2).

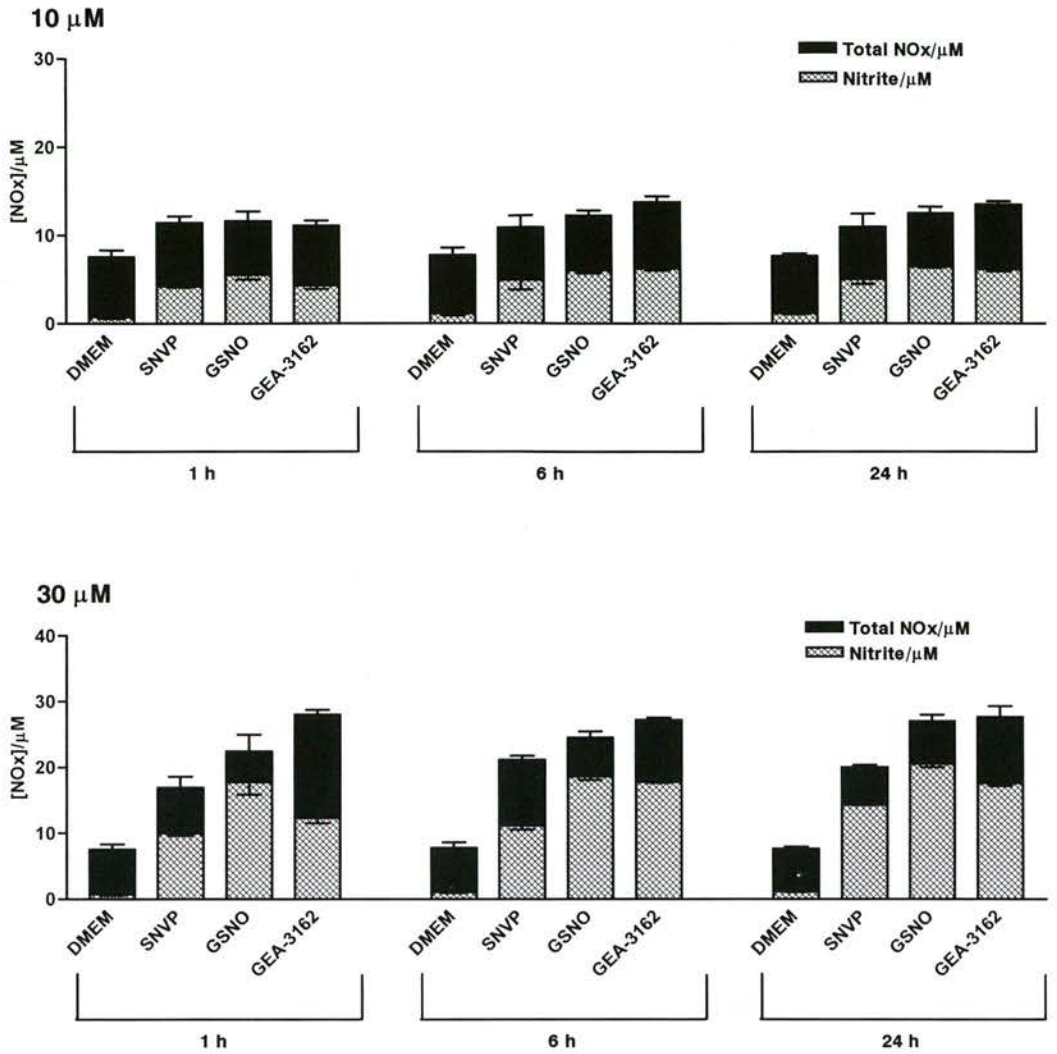

Figure 4.1 NOx Generation by SNVP, GSNO and GEA-3162 in the Presence of BAoSMC

Total NOx production from SNVP, GSNO and GEA-3162 (10 μM and 30 μM) in the presence of BAoSMC was measured by Greiss Test at 1h, 6h, and 24 h time points. The proportion of NO_2^- is indicated by lower, hatched sections of bars. n= 5 for all compounds at all time points.

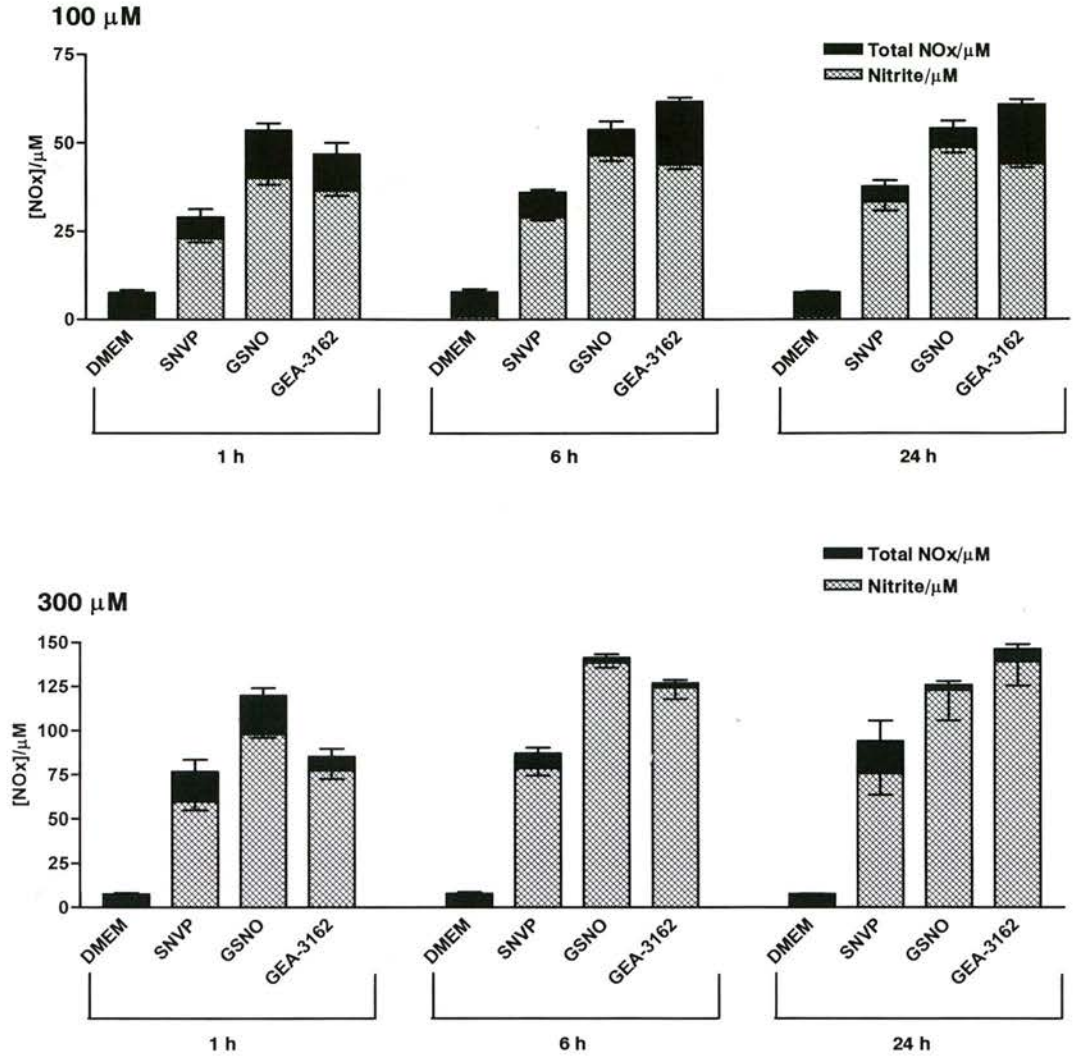

Figure 4.2 NOx Generation by SNVP, GSNO and GEA-3162 in the Presence of BAoSMC

Total NOx production from, SNVP, GSNO and GEA-3162 (100 μM; and 300 μM) in the presence of BAoSMC was measured by Greiss Test at 1h, 6h, and 24 h time points. The proportion of NO_2^- is indicated by lower, hatched sections of bars. n= 5 for all compounds at all time points.

4.3.2 The Effect of NO-Related Species on Cell Proliferation in BAoSMC

The maximum concentration of drug vehicle (DMSO; 1%) had no effect on the level of cell proliferation compared to control, untreated cells (P>0.05; unpaired one-way ANOVA; n=6; figure 4.3). Similarly, the diazeniumdiolate compound, DEA/NO, and the RS-N=O compounds, SNVP and GSNO (all 10 – 300 μM) had no effect of BAoSMC proliferation (P>0.05; unpaired, one-way ANOVA; n=6; figure 4.3). DETA/NO inhibited cell proliferation, but this inhibition only reached significance at the highest concentration tested (300 μM; P<0.05; unpaired, one-way ANOVA followed by post hoc Dunnett's test). The $ONOO^-$ generator, GEA-3162, produced a concentration-dependent decrease in the level of cell proliferation (P<0.01; unpaired, one-way ANOVA followed by post hoc Dunnett's test; figure 4.3; n=6).

The specific sGC inhibitor, ODQ (20 μM), or the O_2^- scavenger, SOD (500 $U.ml^{-1}$), had no further impact on cell proliferation in those NO donor treatments which failed to affect cell proliferation (DEA/NO and the RS-N=O compounds; data not shown). ODQ significantly attenuated the inhibition in cell proliferation induced by DETA/NO (P<0.05; two-way ANOVA; n=6; figure 4.4), but did not effect GEA-3162 induced inhibition of mitogenesis (P>0.05; two-way ANOVA; n=6; figure 4.4). SOD had no effect on the decrease in cell proliferation induced by DETA/NO or GEA-3162 (P>0.05 for both treatments; two-way ANOVA; n=6; figures 4.5).

It was not possible to assess the impact of the NO-specific scavenger, carboxy-PTIO, on cell proliferation as the compound interfered with the BrdU assay resulting in the absence of a signal in all cells treated with carboxy-PTIO.

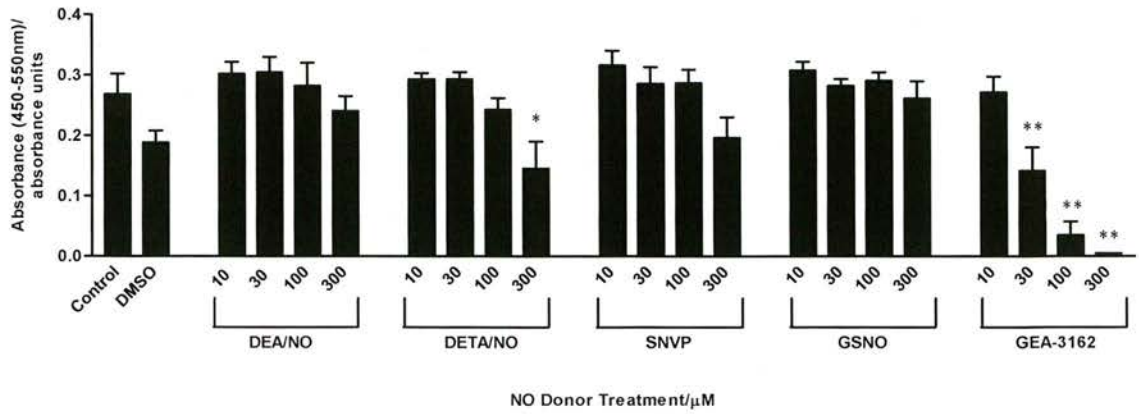

Figure 4.3 The Effect of NO Donor Compounds on BAoSMC Proliferation

The maximum concentration of drug vehicle (DMSO; 1%), DEA/NO, SNVP and GSNO (all 10 – 300 μM) had no significant impact on cell proliferation in BAoSMC compared to control, untreated cells ($P>0.05$; unpaired, one-way ANOVA; n=6). DETA/NO (300 μM) caused a significant reduction in cell proliferation (* = $P<0.05$). GEA-3162 (10 – 300 μM) caused a concentration-dependent decrease in cell proliferation compared to control cells (** = $P<0.01$; unpaired, one-way ANOVA followed by post hoc Dunnett's test; n=6).

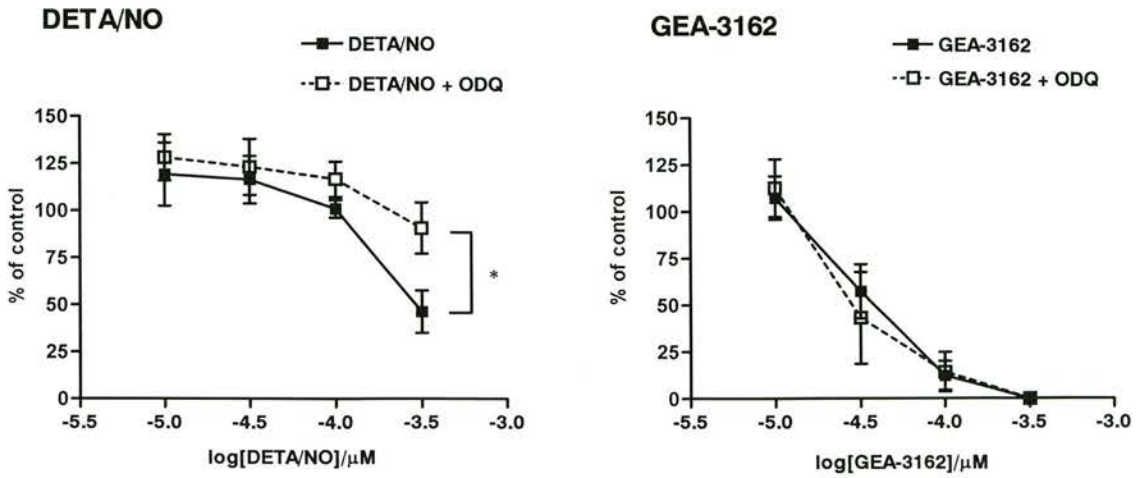

Figure 4.4 The Effect of ODQ on DETA/NO and GEA-3162 Induced Inhibition of Cell Proliferation in BAoSMC

ODQ (20 μM; open symbols, dashed line) significantly attenuated the inhibition of cell proliferation induced by DETA/NO (closed symbols, solid line) but had no effect on GEA-3162 induced inhibition of proliferation (closed symbols, solid line). * = $P<0.05$; two-way ANOVA;

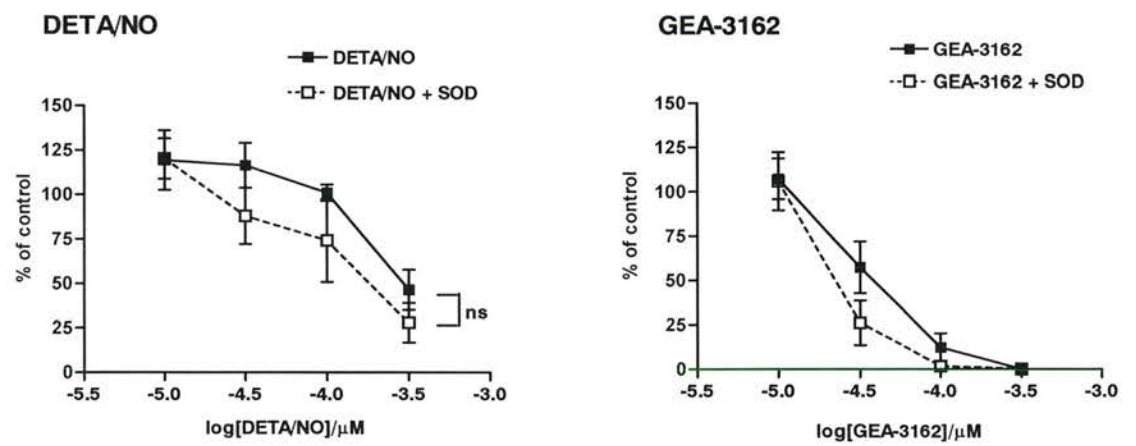

Figure 4.5 The Effect of SOD on DETA/NO and GEA-3162 Induced Inhibition of Cell Proliferation in BAoSMC

SOD (500 $U.ml^{-1}$; open symbols, dashed line) had no effect on the inhibition of cell proliferation induced by DETA/NO (closed symbols, solid line; A;) or GEA-3162 (closed symbols, solid line; B). $P<0.05$ for both treatments; two-way ANOVA; n=6

4.3.3 The Effect of NO-Related Species on Cell Viability in BAoSMC

Although DETA/NO and GEA-3162 appeared to cause a concentration-dependent decrease in cell proliferation, this could in fact be due to cytotoxic properties of the compounds rather than a genuine decrease in proliferation. In order to further investigate this, the cytotoxic impact of all NO donor treatments was assessed in BAoSMC by lactate dehydrogenase (LDH) assay and flow cytometric analysis of cell surface markers of apoptosis and necrosis (annexin V binding to phosphatiylserine (PS) and prodidium iodide (PI) staining respectively).

4.3.3.1 Lactate Dehydrogenase Assay

The maximum concentration of drug vehicle (DMSO; 1%) had no effect on the level of cell viability compared to control, untreated cells ($P>0.05$; unpaired one-way ANOVA; n=6; figure 4.6). Similarly, the diazeniumdiolate compounds, DEA/NO and DETA/NO, and the RS-N=O compounds, SNVP and GSNO (all 10 – 300 μM) had no effect on BAoSMC viability ($P>0.05$; unpaired, one-way ANOVA; n=14; figure 4.5). The $ONOO^-$ generator, GEA-3162, produced a concentration-dependent increase in the level of cytotoxicity compared to control, untreated cells ($P<0.01$; unpaired, one-way ANOVA followed by post hoc Dunnett's test; figure 4.6; n=14).

The O_2^- scavenger, SOD (500 $U.ml^{-1}$), the specific sGC inhibitor, ODQ (20 μM), and specific NO scavenger, carboxyPTIO (500 μM), had no further impact on cytotoxicity in those NO donor treatments which failed to affect cytotoxicity (the diazeniumdiolate and RS-N=O compounds; data not shown). SOD significantly

attenuated the concentration-dependent increase in cytotoxicity induced by GEA-3162 ($P<0.001$; two-way ANOVA followed by Bonferroni post hoc test; n=6; figure 4.7). ODQ and carboxyPTIO had no effect on the GEA-3162 induced cyotoxicity ($P>0.05$ for both treatments; two-way ANOVA; n=6; figure 4.7).

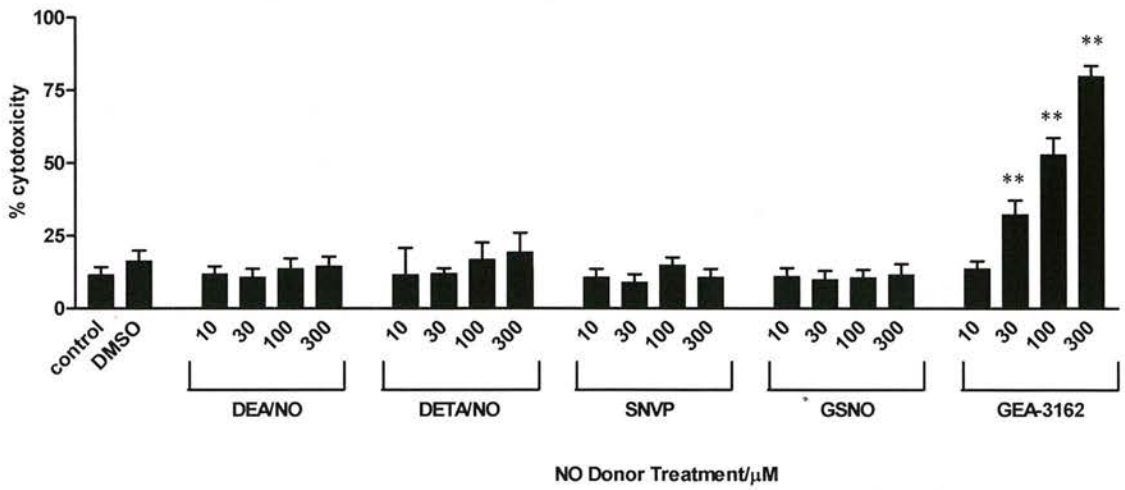

Figure 4.6 Cytotoxic Impact of NO Donor Compounds on BAoSMC

The maximum concentration of drug vehicle (DMSO; 1%), DEA/NO, DETA/NO, SNVP and GSNO (all 10 – 300 μM) had no significant impact on cell viability in BAoSMC compared to control, untreated cells ($P>0.05$; unpaired, one-way ANOVA; n=14). GEA-3162 (10 – 300 μM) caused a concentration-dependent increase in cytotoxicity compared to control cells (** = $P<0.01$; unpaired, one-way ANOVA followed by post hoc Dunnett's test; n=14).

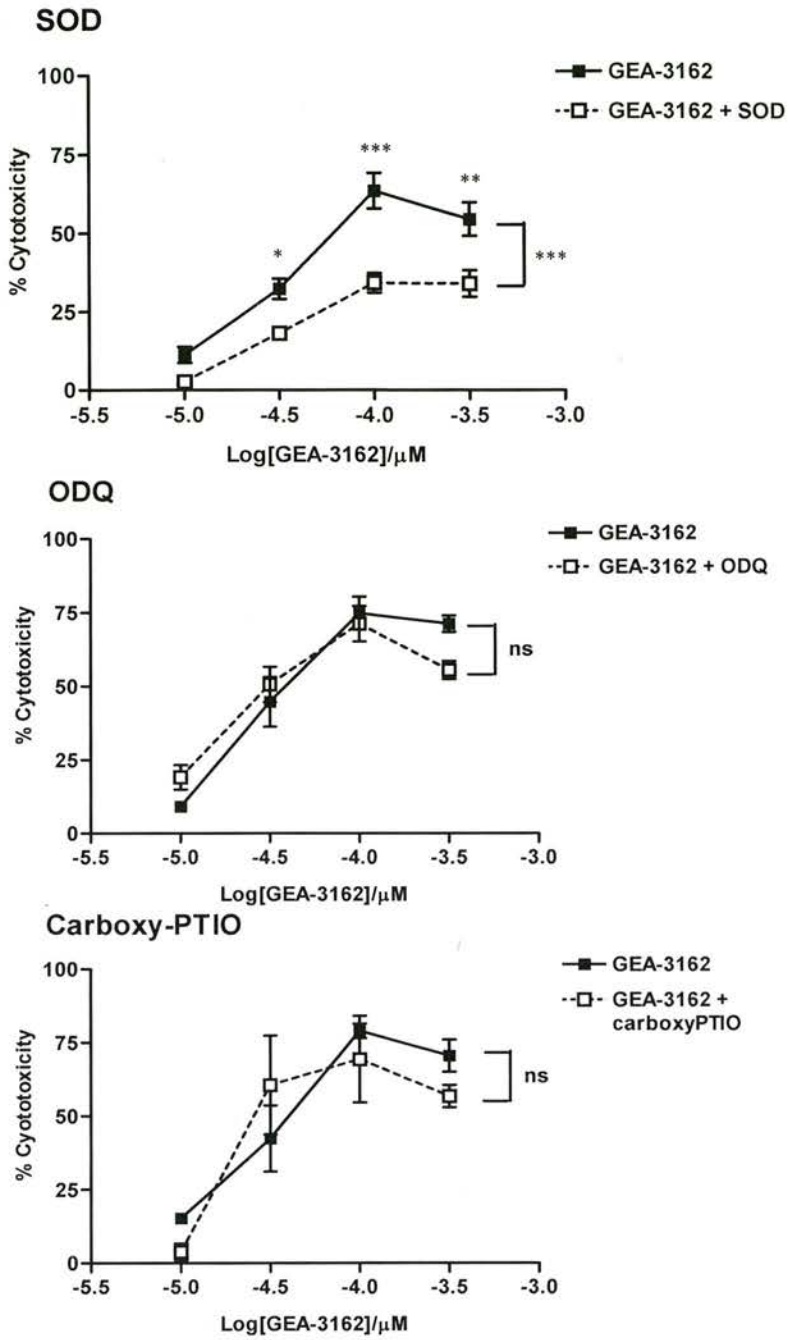

Figure 4.7 The Effect of SOD, ODQ and CarboxyPTIO on GEA-3162 Induced Cytotoxicity in BAoSMC

Cytotoxicity induced by GEA-3162 (10 – 300 μM; closed symbols, solid line) was significantly attenuated by SOD (500 $U.ml^{-1}$; open symbols, dashed line). * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$ (two-way ANOVA followed by post hoc Bonferroni's test; n=6). ODQ (20 μM; open symbols, dashed line) and carboxyPTIO (500 μM; open symbols, dashed line) failed to abrogate GEA-3162 induced cytotoxicity (ns = $P>0.05$; two-way ANOVA; n=8 for ODQ and n=6 for carboxyPTIO).

4.3.3.2 Flow Cytometric Characterisation of Cell Death in BAoSMC

Analysis by flow cytometry confirmed that the maximum concentration of drug vehicle (DMSO; 1%) together with the diazeniumdiolate compounds, DEA/NO and DETA/NO, and the RS-N=O compounds, SNVP and GSNO (all 10 – 300 μM), had no effect on cell viability in BAoSMC. Following both a 6 and 24 h incubation with the above treatments, the level of annexin V binding or PI staining was not significantly altered compared to control, untreated cells (P>0.05 for all treatments at both time points; unpaired, one-way ANOVA; n=6 at 6 h and n=7 at 24 h; figure 4.8).

GEA-3162 (10 – 300 μM) caused a concentration-dependent increase in the number of cells positive for annexin V binding compared to control, untreated cells at both 6 h and 24 h. This increase in annexin V binding occurred in combination with an increase in the number of cells positive for PI staining (P values ranging from <0.05 to < 0.001; unpaired, one-way ANOVA followed by post hoc Dunnett's test; n=6 at 6 h and n=7 at 24 h; figure 4.8).

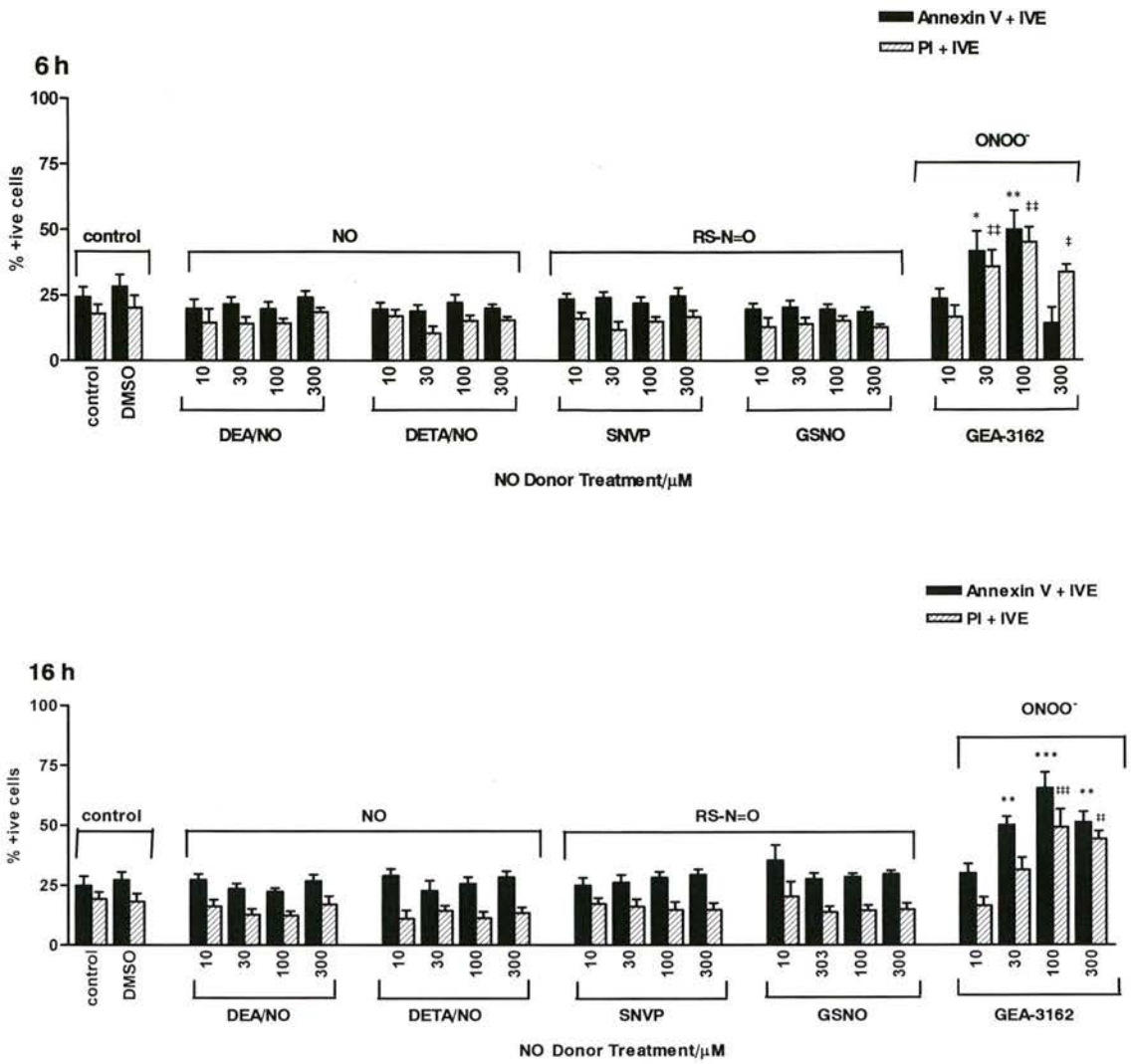

Figure 4.8 Analysis of Cell Death in BAoSMC by Flow cytometry

The maximum concentration of drug vehicle (DMSO; 1%), DEA/NO, DETA/NO, SNVP and GSNO (all 10 – 300 μM) had no significant impact on the number of cells positive for annexin V binding (solid bars) or PI staining (striped bars) following a 6 h or 24 h incubation (P>0.05; unpaired, one-way ANOVA; n=6 at 6 h and n=7 at 24 h). GEA-3162 (10 – 300 μM) caused a concentration-dependent increase in the number cells positive for annexin V binding (* = P<0.05, ** = P<0.01, *** = P<0.001; unpaired, one-way ANOVA followed by Dunnett's post hoc test; n=6 at 6 h and n=7 at 24 h), and this was accompanied by a significant increase in the number of cells positive for PI staining (‡ = P<0.05, ‡‡ = P<0.01, ‡‡‡ = P<0.001; unpaired, one-way ANOVA followed by post hoc Dunnett's test; n=6 at 6 h and n=7 at 24 h).

4.4 Discussion

These studies demonstrate that, contrary to previous reports (Garg and Hassid 1989; Garg and Hassid 1993), RS-N=O compounds have no effect on SMC proliferation or viability. NO radical delivered as a burst of NO is similarly incapable of affecting VSMC proliferation or viability, but a prolonged delivery of NO inhibits cell proliferation in the absence of cytotoxic effects. The NO-related species, $ONOO^-$, appears to inhibit cell proliferation, but further investigation revealed that this is likely to be due to cytotoxic actions of $ONOO^-$, resulting in a loss of cell number due to necrotic cell death.

Interestingly, Griess test measurements showed that SNVP, GSNO and GEA-3162 release the majority of the NO, or NO-related species, during the initial hour of the experiment, with levels of total NOx remaining relatively steady throughout the subsequent incubation period, suggesting that a component of DMEM is accelerating NOx generation. Previous experiments (chapter three) identified that NO release is highly dependent on the chemical composition of the solution into which NO donor compounds are introduced, with release profiles being sensitive to fundamental chemical components, such as buffering means. Although the component(s) of DMEM that might cause this initial acceleration of NO release has not been identified in these studies, the observations further underline the necessity to conduct such preliminary measurements under experimental conditions.

Overall, the results from the Greiss test experiments show that, although each class of compound releases NO in a different chemical form, the total NOx generated is comparable between the RS-N=O compounds and the mesoionic oxatriazole derivative. Although it was not possible to measure NOx generation from

diazeniumdiolate compounds by Griess test, previous experiments (chapter three) demonstrated that DEA/NO and DETA/NO release NO radical as would be predicted from their respective half-lives – a rapid and short burst of NO from DEA/NO ($t_{1/2}$ = 2 min), and a prolonged and sustained generation of NO from DETA/NO ($t_{1/2}$ = 20 h).

Despite previous reports demonstrating that NO, NO-related species, and agents that act to elevate cGMP are capable of inhibiting VSMC proliferation (Garg and Hassid 1989; Kariya et al. 1989; Assender et al. 1992; Newby et al. 1992; Garg and Hassid 1993; Mooradian et al. 1995; Yu et al. 1997; Jeremy et al. 1999), the results presented here show that, of a range of compounds capable of releasing various NO and NO-related species, only a slow, prolonged delivery of NO radical or $ONOO^-$, has an inhibitory effect on cell proliferation. Comparable concentrations of NOx delivered by RS-N=O compounds, or higher concentrations delivered as rapid burst of NO radical by a short acting diazeniumdiolate compound, are incapable of directly effecting VSMC proliferation or viability.

Inherent to anti-mitogenesis studies are the difficulties arising from cytotoxicity of compounds. An apparent inhibition of proliferation may, in fact, be due to decreasing cell number as a result of cytotoxic actions of a compound. Given that GEA-3162 induces necrotic cell death (evidenced by LDH release and elevated levels of PI staining) over the same concentration range as the apparent inhibition of proliferation, it is likely that this is not a genuine inhibition of mitogenesis, but a loss of overall cell number. However, whilst GEA-3162-induced cytotoxicity was attenuated by SOD, the decrease in proliferation induced by this compound was insensitive to SOD. This could indicate a residual, genuine anti-mitogenic effect

being masked by the cytotoxic properties of GEA-3162. However, as cGMP is widely accepted to be the downstream effector of inhibition of proliferation (Garg and Hassid 1989; Kariya et al. 1989; Assender et al. 1992), the specific sGC inhibitor, ODQ, would be expected to attenuate any remaining anti-mitogenic effects. This was not seen and ODQ had no effect of the apparent GEA-3162-induced anti-mitogenic effects. Alternatively, cGMP-independent pathways could be responsible for any underlying cytostatic effects, however, considered in combination with the LDH assay and flow cytometry results, these data strongly suggest that $ONOO^-$ is cytotoxic, rather than genuinely anti-mitogenic. This potential difficulty in interpreting the data from anti-mitogenesis studies has, to some extent, been overlooked by previous investigators. For example, RS-N=O compounds have been demonstrated to inhibit SMC proliferation but this occurred at higher concentrations of compound than used here (in the mM range) and interestingly, was attenuated by SOD, suggesting that O_2^-, or $ONOO^-$, is ultimately effector of this inhibition. It seems likely that this observed inhibition of proliferation could, therefore, be due to cytotoxicity of $ONOO^-$ formed as an intermediate species, which was not explored fully (Garg and Hassid 1989). Similarly, SIN-1, which is structurally related to GEA-3162 and now accepted to be a $ONOO^-$ generator, has been reported to inhibit rabbit SMC mitogenesis, but no investigation into the cytotoxicity of this compound was conducted (Assender et al. 1992). The demonstration that an apparent inhibition of proliferation by GEA-3162 was, in fact, a result of necrotic cell death underlines the absolute necessity to conduct cytotoxicity studies in conjunction with mitogenesis studies.

That cytotoxicity induced by $ONOO^-$ in BAoSMC was characterised by flow cytometry to be necrosis, rather than apoptosis is perhaps unsurprising, as the cytotoxic actions of NO have often been ascribed to non-specific oxidative chemistry. However, $ONOO^-$ has previously been demonstrated to induce apoptosis in rat VSMC (Li et al. 2003; Li et al. 2004). There are several possible explanations as to why this phenomenon has not been demonstrated here. Firstly, the methods used to characterise apoptosis in the published studies quoted above (trypan blue exclusion and TUNEL staining) are not as sensitive or as specific as the flow cytometry methods employed in this study, where markers of both apoptosis and necrosis were examined in the same cell. Secondly, the ability of an agent to induce apoptosis, rather than necrosis, in VSMC may be dependent on the precise phenotype of the VSMC. A recent study has highlighted significant differences between the ability of certain NO donors to induce apoptosis in VSMC of varying phenotypic origin (Lau 2003). Although apoptosis was induced by sodium nitroprusside (SNP) or the RS-N=O, *S*-nitroso-*N*-acetylpenicillamine (SNAP), rather than $ONOO^-$, the study by Lau emphasises how specifically apoptosis is regulated and suggests that VSMCs are more susceptible to apoptotic cell death when they are of the proliferative phenotype. Cells of a proliferative phenotype make up only a small percentage of the VSMC population in healthy vessels, but come to dominate following the phenotypic alterations that occur during vascular injury (including atherosclerosis; Lau 2003). The cells examined in the current study were a bovine aortic smooth muscle cell line harvested from the aortae of healthy animals. Although VSMCs undergo phenotypic alteration during culturing (Absher et al.

1989), it is not clear whether they develop a proliferative phenotype that is susceptible to apoptotic stimuli.

Given the cytotoxic properties of $ONOO^-$, it is likely to have serious adverse consequences for the atherosclerotic plaque. Due to the environment of elevated oxidative stress, or more likely, due to a reduction in the anti-oxidant capacity of the cell during atherosclerosis, any available NO, either endothelium-derived or delivered by synthetic compounds, is likely to react rapidly with O_2^- to form $ONOO^-$ in situ. The cytotoxic properties of $ONOO^-$ would then result in a loss of cells from the VSMC population of the plaque cap, increasing the likelihood of rupture. Plaque rupture is associated with, and considered to be the underlying cause of, the acute clinical consequences of atherosclerosis including myocardial infarction and stroke (Davies 1995; Schroeder and Falk 1995; Dalager-Pedersen et al. 1998; Gutstein and Fuster 1999; Zhou et al. 1999; Corti and Badimon 2002; Mitra et al. 2004).

Although the apparent anti-proliferative actions of $ONOO^-$ can largely be ascribed to cytotoxicity, this is not the case for the anti-mitogenic actions of DETA/NO, which was non-cytotoxic towards BAoSMC. The specific sGC inhibitor, ODQ, partially attenuated DETA/NO-induced inhibition of proliferation, demonstrating that these actions are, at least partly, cGMP-dependent. The precise mechanism by which cyclic nucleotides bring about inhibition of proliferation remains a source of some debate and likely involves multiple complex signalling pathways. cGMP activates protein kinase G (PKG) which can then trigger signalling pathways involving p42/44 mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERK), leading ultimately to decreases in DNA synthesis (Bauer et al. 2001; Koyama et al. 2001). Although ODQ significantly

inhibited the cytostatic effects of DETA/NO, this inhibition was partial and a residual inhibition of proliferation remained in the face of ODQ treatment. This could potentially be due to incomplete inhibition of sGC, but this is unlikely as the concentration of ODQ used in these studies has been demonstrated to be effective during attenuation of NO-dependent inhibition platelet aggregation (Crane et al. 2005). It is therefore more likely that there is a cGMP-independent component to anti-proliferative actions of DETA/NO. cGMP-independent pathways have been implicated previously in the cytostatic actions of DETA/NO in rat aortic VSMC (Ignarro et al. 2001), although the mechanism of such pathways remains to be identified.

The observation that a burst of NO delivered by DEA/NO is insufficient to inhibit cell proliferation in VSMC is in keeping with the findings of Mooradian *et al* in a study into the effect on proliferation of the rate of release of NO (Mooradian et al. 1995). Using a range of diazeniumdiolate compounds with varying half-lives, Mooradian *et al* reported that DEA/NO and PAPA/NO ($t_{1/2}$ = 15 min) had no effect on proliferation, but that a slower, prolonged delivery achieved by use of DETA/NO caused significant inhibition of rat aortic SMC proliferation, with the maximum inhibition occurring following treatment for 3-5 days with a non-toxic concentration (500 μM) of DETA/NO (Mooradian et al. 1995). The study by Mooradian *et al*, together with the results presented in the current study, demonstrate that the total concentration of NO delivered is not the critical factor in determining cytostatic effects; rather the NO must be delivered over a prolonged period in order to sustain cGMP concentrations at levels sufficient to inhibit proliferation. Therefore, short acting diazeniumdiolate compounds fail to inhibit proliferation due to rapid decline

in cGMP levels following the short burst of NO. Based on NO electrode recordings (chapter three), a 300 μM concentration of DEA/NO can be crudely calculated to release a peak NO concentration in region of 330 μM which would take approximately 40 minutes to decay to nM range, whilst the same concentration of DETA/NO might produce a peak release of approximately only 4 μM NO, but this would take in the region of 180 hours to decay to similar nM concentrations. cGMP production is thought to be triggered by NO in 5-100 nM range, with the half-life of activation being 1-2 seconds (Condorelli and George 2001). Therefore, DETA/NO will continue to generate levels of NO capable of sustaining cGMP production for significantly longer than DEA/NO. The observation that cGMP production must be sustained over a relatively long period, considered in combination with the Greiss test results suggesting a component of DMEM accelerates the rate of NOx generation from RS-N=O compounds, may explain why these compounds failed to inhibit cell proliferation. As already discussed, the RS-N=O compounds generated the total level of NOx during the first hour of delivery. Therefore, these compounds may not stimulate sGC adequately to provide sufficiently elevated cGMP levels over a long enough period to inhibit proliferation. That duration of cGMP production is the critical factor for producing cytostatic effects is further evidenced by the observation that inhibitors of PDEs (the enzyme responsible for cGMP breakdown), including the cGMP-specific PDE-V inhibitor, Sildenafil, also possess anti-proliferative properties in human pulmonary SMC (Tantini et al. 2005).

Of the compounds examined in these studies, the prolonged release of relatively low concentrations of NO from DETA/NO could be considered to be the most realistic model of physiological NO production by the healthy endothelium. In

healthy blood vessels the NO released from the endothelium doubtless acts to prevent uncontrolled proliferation of VSMCs, thus maintaining the vessel wall and preventing hypertrophy. During atherosclerosis, the net production of NO decreases and vascular remodelling occurs, resulting in a thickening of the vessel wall, to which the removal of NO-mediated inhibition of proliferation may contribute. During the later stages of atherosclerosis, when the vessel lumen can expand no further to accommodate the growing lesion, this thickening of the vessel wall may contribute to stenosis of the vessel. However, during the earlier stages of atherogenesis, removing an inhibition of VSMC proliferation may be an initial protective mechanism to maintain the integrity of the plaque cap, and hence prevent lesion rupture.

In summary, the salient finding of these studies, is that, of a range of compounds capable of releasing NO and various NO-related species, only the slow release of NO radical is sufficient to cause inhibition of VSMC proliferation in the absence of cytotoxicity. $ONOO^-$ is the only NO-related species to be cytotoxic towards VSMC, inducing necrosis, but not apoptosis.

Chapter Five

The Effect of NO-Related Species on the Cell Viability of Human Monocyte-Derived Macrophages

5. The Effect of NO and NO-Related Species on the Cell Viability of Human Monocyte-Derived Macrophages

5.1 Introduction

Apoptosis is a fundamental process governing cell survival and differs from necrosis in that it is an active, regulated process in which the cell is required to expend energy prior to cell death. During apoptosis the cell undergoes a number of characteristic morphological changes including membrane blebbing, chromatin condensation, nuclear fragmentation and cell shrinkage (Uren and Vaux 1996; Wyllie 1997). The externalisation of cell surface markers, such as phosphatidylserine (PS), on the outer cell membrane, allows recognition of apoptotic cells by phagocytes, leading ultimately to their clearance (Savill et al. 1989; Fadok et al. 1992; Savill et al. 1993; Fadok et al. 2001; Hoffmann et al. 2001; Huynh et al. 2002). A crucial difference between apoptosis and necrosis is the preservation of the intact cellular membrane. During the apoptotic process the cell membrane is maintained, preventing the release of the cell contents into the surrounding tissue (Meagher et al. 1992; Stern et al. 1996; Lawrence et al. 2001). Apoptosis of inflammatory cells, therefore, represents a non-inflammatory mechanism for the removal of cells from a site of tissue damage, thus preventing the exacerbation of inflammatory responses which would occur if the histotoxic, pro-inflammatory mediators contained within the cell were released (Haslett 1997; Maderna and Godson 2003; Taylor et al. 2003; Rossi et al. 2004). Hence, apoptosis of

inflammatory cells is critical to ensuring the successful resolution of the inflammatory response. Failure of inflammatory cells to undergo apoptosis, or failure of phagocytic removal of apoptotic cells, can lead to incomplete resolution of the inflammatory response because apoptotic cells remaining *in situ* undergo secondary necrosis, resulting in rupture of the cell membrane, and the subsequent release of the pro-inflammatory cell contents (Haslett 1997). Pharmacological manipulation of apoptosis may aid the resolution of inflammation occurring in chronic inflammatory disorders and, therefore, represents a novel therapeutic strategy for the treatment of such conditions (Ward et al. 1999; Gilroy et al. 2004).

Atherosclerosis is now considered to be the consequence of a chronic, low-grade inflammatory response (Ross 1999a; Ross 1999b; Berk et al. 2001; Libby 2002; Libby et al. 2002; Robbins and Topol 2002). Lipid laden macrophages, or foam cells, retained in the vessel wall are the major constituent of the plaque core (Ross 1993; Davies 1997) and recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind plaque growth and development. Given that apoptosis of inflammatory cells results in their non-inflammatory phagocytosis, inducing apoptosis in macrophages within the plaque core may aid resolution of vascular inflammation, and could potentially limit disease progression, or even aid plaque regression.

NO is a possible candidate for use in the manipulation of apoptosis during atherosclerosis. NO has previously been demonstrated to induce apoptosis and aid subsequent inflammatory resolution in several animal models (Duffield et al. 2000; Duffield et al. 2001; Saio et al. 2001; Chattopadhyay et al. 2002), including a rabbit model of atherosclerosis (Wang et al. 1999). Although the production of NO by

human macrophages remains debatable, such cells do respond to exogenous NO delivered by synthetic NO donor compounds, indicating the potential of NO to induce apoptosis in macrophages (Albina 1995; Weinberg et al. 1995; Thomassen and Kavuru 2001; Schneemann and Schoedon 2002). The downstream effectors of NO-induced apoptosis are not yet fully identified but are likely to be multiple, cell type-specific, and are generally regarded to occur independently of cGMP signalling (Nicotera et al. 1997; Garban and Bonavida 1999; Wang et al. 1999; Manderscheid et al. 2001; Boyle et al. 2002). For example, NO may induce down-regulation of intracellular caspase inhibitors such as the inhibitors of apoptosis proteins (IAPs; Manderscheid et al. 2001), and/or increase the sensitivity of cells to apoptotic stimuli such as Fas ligand (FasL; Garban and Bonavida 1999; Boyle et al. 2002)

Considered in combination with additional powerful anti-atherogenic characteristics of NO, including inhibition of platelet activation (Deana et al. 1989; Radomski et al. 1992) and various other anti-inflammatory properties (Tsao et al. 1994; De Caterina et al. 1995; Zeiher et al. 1995; Tomita et al. 1998; Qian et al. 1999), the potential beneficial pro-apoptotic actions of NO make this molecule an appealing prospect as a means of regressing atherosclerotic plaques. However, the success of such a therapeutic strategy relies on the ability to deliver NO with some degree of selectivity in order to specifically target monocytes or macrophages within the core, without triggering vascular collapse or indiscriminate pro-apoptotic events in other cell types present in the plaque. For example, apoptosis of the VSMC population in the plaque cap could have serious detrimental consequences, resulting in plaque rupture (Bauriedel et al. 1999; Leskinen et al. 2003). A possible means to achieve this necessary selectivity may be to exploit differences between the actions

of NO-related species, delivering NO in a form that will induce apoptosis in one cell type, whilst leaving other cell types unaffected. The main aim of these studies was therefore to test the hypothesis that various NO-related species would differentially induce apoptosis in human macrophages. A further aim was to examine the ability of human monocyte-derived macrophages to generate NO in order to understand some of the *in vivo* mechanisms underlying atherosclerosis.

5.2 Methods

5.2.1 Cell Culture

5.2.1.1 THP-1 Cell Culture

THP-1 cells (a human macrophage cell line) were cultured as described in chapter two (section 2.2.1).

5.2.1.2 Isolation and Culture of Human Monocyte-Derived Macrophages

Human monocytes were isolated from peripheral blood of healthy volunteers as described in chapter two (section 2.2.2). Experiments on monocytes were performed following culture (37 °C, 95% O_2: 5% CO_2) for 24 hr after the initial isolation. Alternatively, experiments on human monocyte-derived macrophages were performed following culture for 5-7 days to allow monocyte differentiation into monocyte-derived macrophages. At the end of these incubation periods the IMDM was aspirated from the wells and replaced with fresh IMDM containing experimental treatments.

5.2.2 Detection of NO_2^-/NO_3^-

5.2.2.1 Detection of NO_2^-/NO_3^- by Griess Test

In order to measure NO_2^-/NO_3^- (NOx) generated by SNVP, GSNO and GEA-3162 (all 10 – 300 μM) in the presence of cells, human monocytes were allowed to differentiate into monocyte-derived macrophages in 48-well, flat-bottomed, tissue culture plates for 5-7 days in IMDM (37°C, 95% O_2: 5% CO_2; section 2.2.2). At the end of this period, the IMDM was aspirated from the wells and replaced with phenol red-free IMDM (containing the same supplements, including 10% autologous serum, as described in section 2.2.2.1) plus each NO donor compound. This was necessary because the phenol red in IMDM gives the medium a colour that absorbs light at the same wavelength as the Greiss test, whereas as phenol red-free IMDM is colourless.

The total NOx concentration generated by SNVP, GSNO and GEA-3162 in IMDM in presence of human monocyte-derived macrophages was measured at 1, 6, and 24 h time points by Griess test (R&D Systems, Abington, U.K) as described in chapter two (section 2.1.2). It was not possible to measure NOx generation from DEA/NO and DETA/NO by Griess test because reagents are acidified during the protocol and NO release from diazeniumdiolate compounds is pH dependent (Davies et al. 2001).

In order to measure NOx generated by human monocyte-derived macrophages, monocytes were allowed to differentiate into monocyte-derived macrophages for 5-7 days. At the end of this period, IMDM was aspirated from the wells and replaced with phenol red-free IMDM (plus supplements, including 10% autologous serum). Cells were treated (24 h) in the absence and presence of lipopolysaccharide (LPS; 1 $\mu g.ml^{-1}$) in combination with the specific iNOS inhibitor,

N-(3-(aminomethyl)benzyl) acetamidine dihydrochloride (1400W; 50 μM; Garvey et al. 1997) or the non-specific NOS inhibitor, N-monomethyl-L-arginine (L-NMMA; 200 μM). Following the 24 h treatment period, supernatants were aspirated from the wells and the concentration of NOx measured by Greiss test described in chapter two (section 2.1.2).

5.2.2.2 Detection of NO_2^- by Fluorescence High Performance Liquid Chromatography

NO_2^- generated by human monocyte-derived macrophages at concentrations below the limit of detection of the Griess test (< 3 μM) were measured by fluorescence high-performance liquid chromatography (HPLC) as described in chapter two (section 2.1.3). Following differentiation, human monocyte-derived macrophages were treated (24 h) in the absence and presence of LPS (1 $\mu g.ml^{-1}$) in combination with 1400W (50 μM) or L-NMMA (200 μM) in phenol red-free IMDM (plus supplements). Following treatment with LPS, supernatants were aspirated from the wells and the concentration of NO_2^- measured by HPLC as described in chapter two (section 2.1.3).

5.2.3 Assessment of Cell Viability

5.2.3.1 Cell Morphology

Cell morphology, as an indicator of cell viability, was assessed in THP-1 cells as described in chapter two (section 2.3.1). Cells were cultured (37°C, 95% O_2: 5% CO_2) in RPMI tissue culture medium (containing supplements described in chapter two (section 2.2.1), including 10% heat-inactivated FSC and treated (2, 4, 6,

16 h) in the absence and presence of the maximum concentration of drug vehicle (DMSO; 1%), DEA/NO, SNVP, GEA-3162 (all 100 μM), or the acknowledged pro-apoptotic agent, gliotoxin (1 $\mu g.ml^{-1}$). To investigate the influence of protein on cell viability, the experiments were repeated at 6 h and 16 h time points in medium containing FCS (10%) and FCS-free medium.

5.2.3.2 Flow Cytometry

Cell death induced by NO donor compounds was characterised in human monocytes and monocyte-derived macrophages as either apoptosis or necrosis by flow cytometry as described in chapter two (section 2.3.3). Cells were cultured (37°C, 95% O_2: 5% CO_2) in IMDM (containing supplements described in section 2.2.2.1, including 10% autologous serum), and treated (24 h) in the absence and presence of the maximum concentration of drug vehicle (DMSO; 1%), DEA/NO, DETA/NO, SNVP, GSNO and GEA-3162 (all 100 – 300 μM), or gliotoxin (1 $\mu g.ml^{-1}$) prior to flow cytometric analysis of cell surface phosphatidylserine (PS) expression (FITC-conjugated annexin V binding) and cell membrane integrity (propidium iodide (PI) staining) as described in chapter two (section 2.3.3). In order to assess the influence of oxidising radical species on the cytotoxic effects of NO donor treatment, the experiments were repeated in the presence of SOD (500 $U.ml^{-1}$)

5.3 Results

5.3.1 NOx Generation By NO Donor Compounds

The concentration of total NOx generated in the presence of human monocyte-derived macrophages was similar for GSNO and GEA-3162, and this was

slightly greater than that generated by SNVP (figures 5.1 & 5.2). This general trend occurred at all concentrations and time points, but was more pronounced at higher concentrations (100 μM and 300 μM). Statistical analysis by unpaired, one-way ANOVA followed by post hoc Tukey test revealed a high degree of variability with no overall clear pattern of significance differences between compounds (analyses not shown). The proportional of total NOx consisting of nitrate (NO_3^-) was variable and tended to be approximate a quarter to half for each compound.

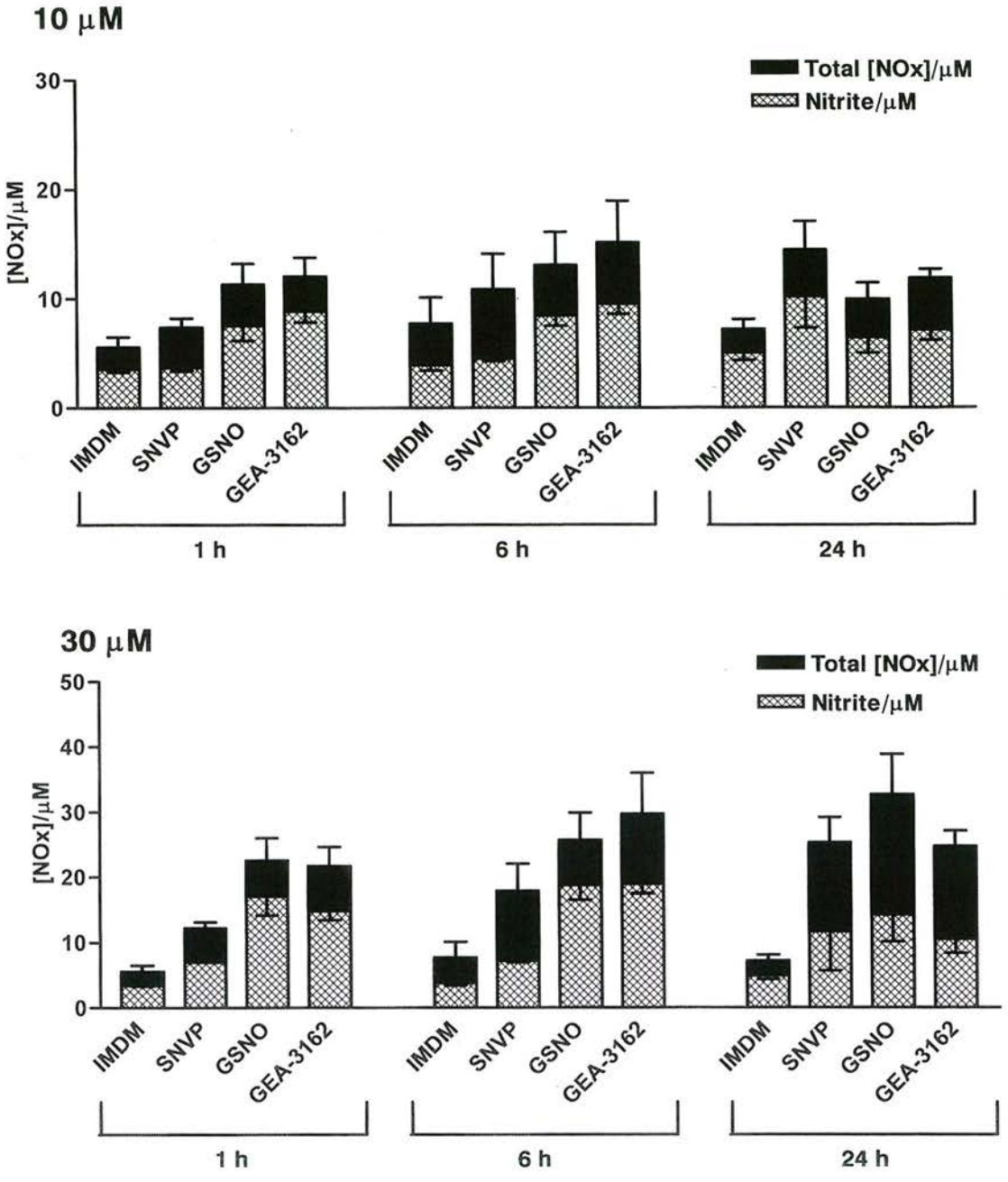

Figure 5.1 NOx Generation by SNVP, GSNO and GEA-3162 in the Presence of Human Monocyte-Derived Macrophages

Total NOx production by SNVP, GSNO and GEA-3162 (10 μM and 30 μM) in the presence of human monocyte-derived macrophages was measured by Greiss test at 1, 6, and 24 h time points as indicated. The proportion of total NOx consisting of NO_2^- is indicated by lower, hatched sections of bars. n= 5 for all compounds at each time point.

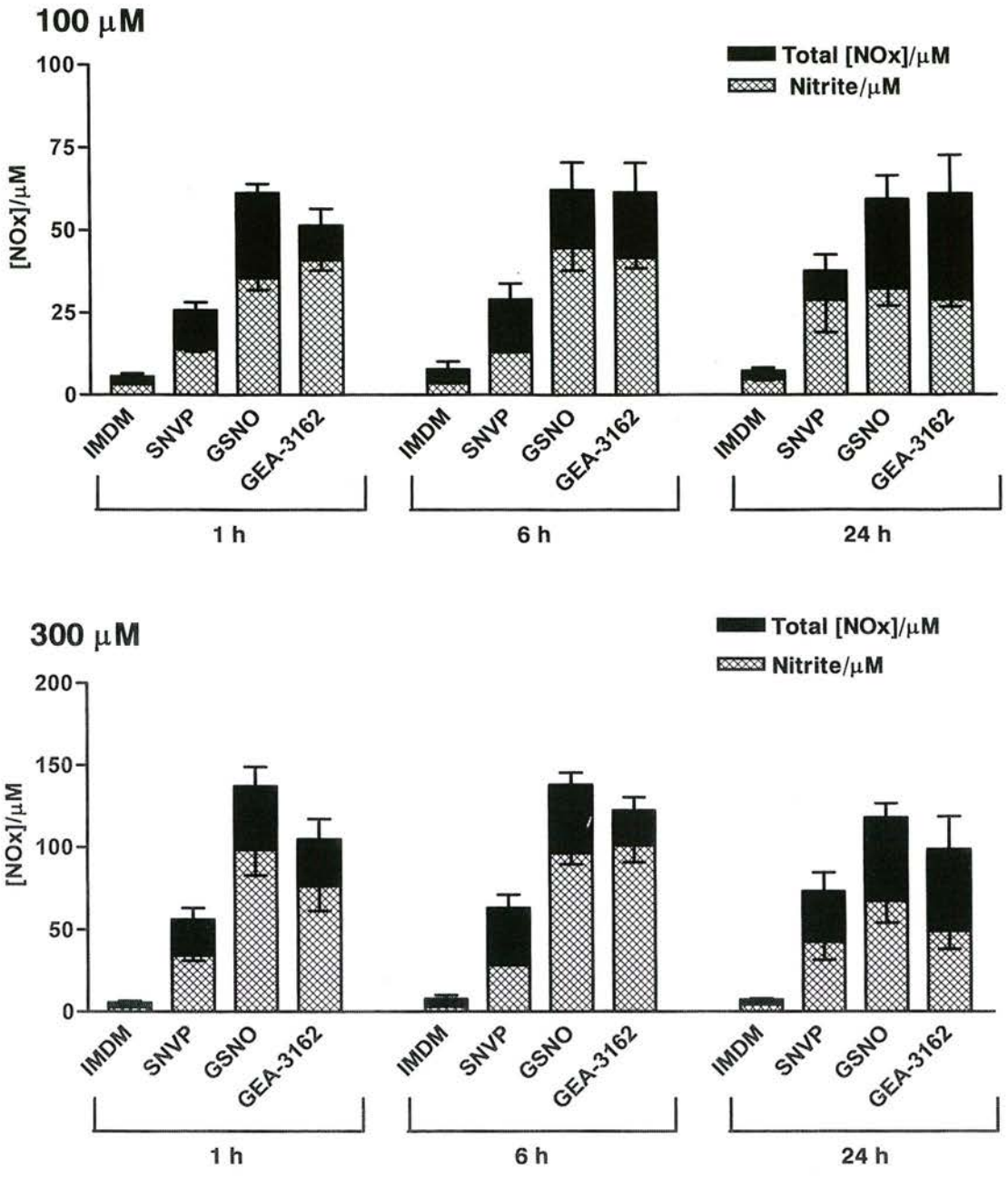

Figure 5.2 NOx Generation by SNVP, GSNO and GEA-3162 in the Presence of Human Monocyte-Derived Macrophages

Total NOx production from SNVP, GSNO and GEA-3162 (100 μM and 300 μM) in the presence of human monocyte-derived macrophages was measured by Greiss Test at 1, 6, and 24 h time points as indicted. The proportion of total NOx consisting of NO_2^- is indicated by lower, hatched sections of bars. n=5 for all compounds at each time point.

5.3.2 NOx Generation by Human Monocyte-derived Macrophages

5.3.2.1 Detection of NOx Greiss Test

NOx generated by human monocyte-derived macrophages was at the lower limit of detection for the Greiss test. LPS (1 $\mu g.ml^{-1}$)-stimulated human monocyte-derived macrophages did not produce significantly greater levels of NOx compared to control (un-stimulated), monocyte-derived macrophages (4.1 ± 1.4 *vs* 4.5 ± 1.0 μM; $P>0.05$; matched, one-way ANOVA; n=5). The level of NOx generated by monocyte-derived macrophages was unaffected by treatment with 1400W (50 μM) or L-NMMA (200 μM; data not shown).

5.3.2.2 Detection of NO_2^- by Fluorescence High Performance Liquid Chromatography

As with the Greiss test, levels of NO_2^- detected from un-stimulated monocyte-derived macrophages were at the lower limit of detection by HPLC (2.2 ± 1.4 nM). It was not possible to make a comparison with NOx generated by LPS-stimulated (1 $\mu g.ml^{-1}$) monocyte-derived macrophages, as NO_2^- was not detected under these conditions (data not shown).

5.3.3 The Effect of NO and NO-Related Species on Cell Viability

5.3.3.1 The Effect of NO-Related Species on Cell Viability Assessed by Morphology in THP-1 Macrophages

The maximum concentration of drug vehicle (DMSO; 1%) had no affect on cell death in THP-1 macrophages compared to untreated cells following 2, 4, 6, or 16 h incubation (figures 5.3). DEA/NO and SNVP (100 μM) did not induce significant cell death in THP-1 macrophages compared to vehicle treated cells at 2, 4 or 6 h incubation ($P>0.05$; one-way ANOVA; n=4 at 2 h, n=5 at 4 h and n=6 at 6 h; figures 5.3 & 5.4). GEA-3162 (100 μM) induced significant levels of cell death compared to vehicle treated cells at 4 and 6 h incubation (5.0 ± 1.7 *vs* 38.5 ± 8.4 %; n=5 at 4 h and 15.5 ± 8.4 *vs* 59.5 ± 12.4 %; n=6; $P<0.01$ at both time points; one-way ANOVA followed by post hoc Dunnett's test; figure 5.3). Following 16 h incubation, DEA/NO, SNVP and GEA-3162 all induced significant levels of cell death compared to vehicle treated cells. The maximum concentration of drug vehicle (DMSO; 1%) induced 14.3 ± 3.4% cell death at 16 h compared to 60.4 ± 7.0 % induced by DEA/NO ($P<0.01$), 44.4 ± 17.4% by SNVP ($P<0.05$) and 81.1 ± 10.2% GEA-3162 ($P<0.01$; one-way ANOVA followed by post hoc Dunnett's test; n=6 for all treatments; figure 5.3). The apoptotic agent, gliotoxin (1 $\mu g.ml^{-1}$), induced significant cell death compared to vehicle treated cells at 6 and 16 h incubation (15.5 ± 8.4 *vs* 50.7 ± 10.9%; $P<0.05$ at 6 h and 14.3 ± 3.4 *vs* 90.8 ± 5.9%; $P<0.01$ at 16 h; unpaired, one-way ANOVA followed by post hoc Dunnett's test; n=6 at all time points; figure 5.3).

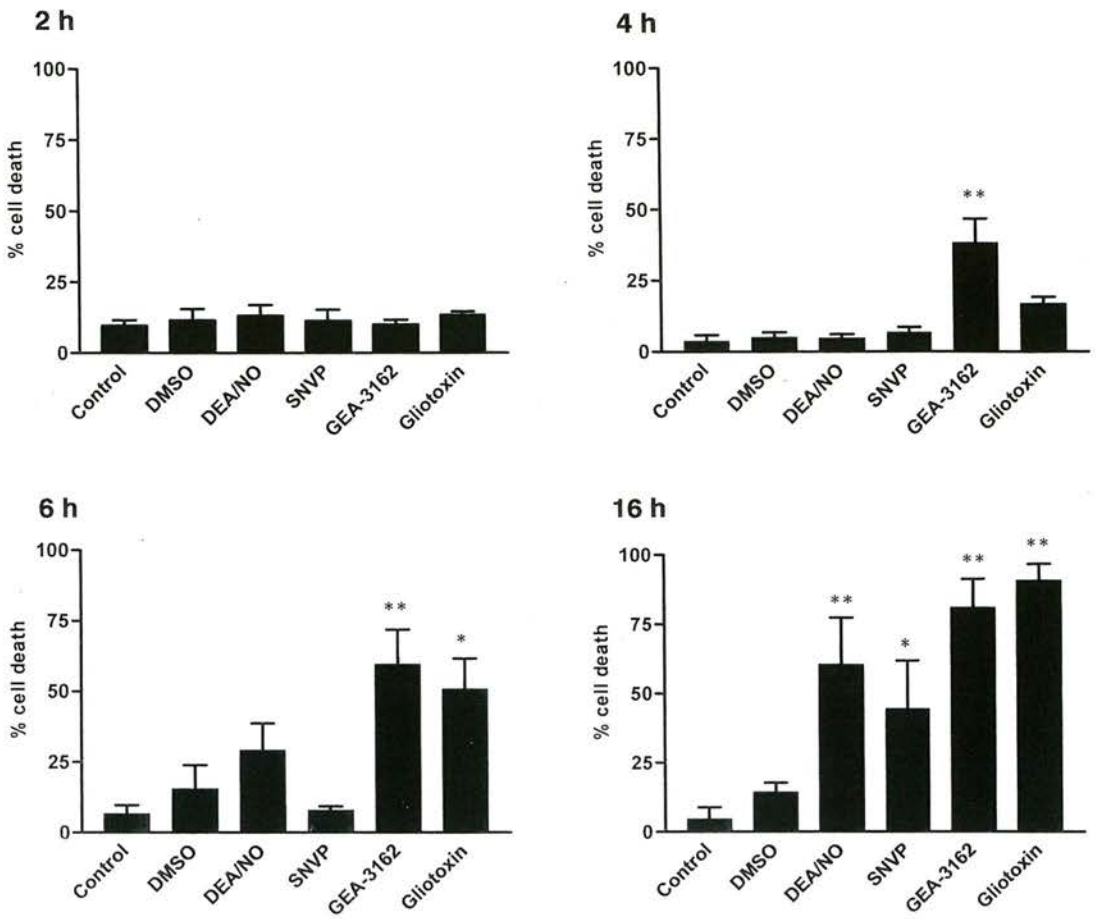

Figure 5.3 Cell Death Induced by NO Donor Compounds in THP-1 Macrophages

Cell death induced by drug vehicle (DMSO; 1%), DEA/NO, SNVP, GEA-3162 (all 100 μM) and gliotoxin (1 $\mu g.ml^{-1}$) was assessed in THP-1 cells by cell morphology following treatment for 2 h, 4 h, 6 h, and 16 h as indicated. Analysis by unpaired, one-way ANOVA followed by post hoc Dunnett's test revealed GEA-3162 induced significant cell death compared to vehicle treated cells at 4, 6, and 16 h (** = $P<0.01$), DEA/NO and SNVP induced significant cell death at 16 h (** = $P<0.01$ and * = $P<0.05$), and gliotoxin induced significant cell death at 6 and 16 h (** = $P<0.01$). n=4 at 2 h, n=5 at 4, n=6 at 6 and 16 h.

In order to investigate the influence of protein in the medium, the experiments were repeated at 6 and 16 h incubation in presence and absence of heat inactivated foetal calf serum (FCS; 10%). The absence of serum in the medium did not significantly alter the level of cell death induced by any treatment at either time point ($P>0.05$; unpaired one-way ANOVA; n=6 for all treatments at both timepoints). The pattern of cell death was unchanged from that described above, with DEA/NO and SNVP causing significant cell death following a 16 h incubation, and GEA-3162 and gliotoxin being cytotoxic at earlier time points (figure 5.4).

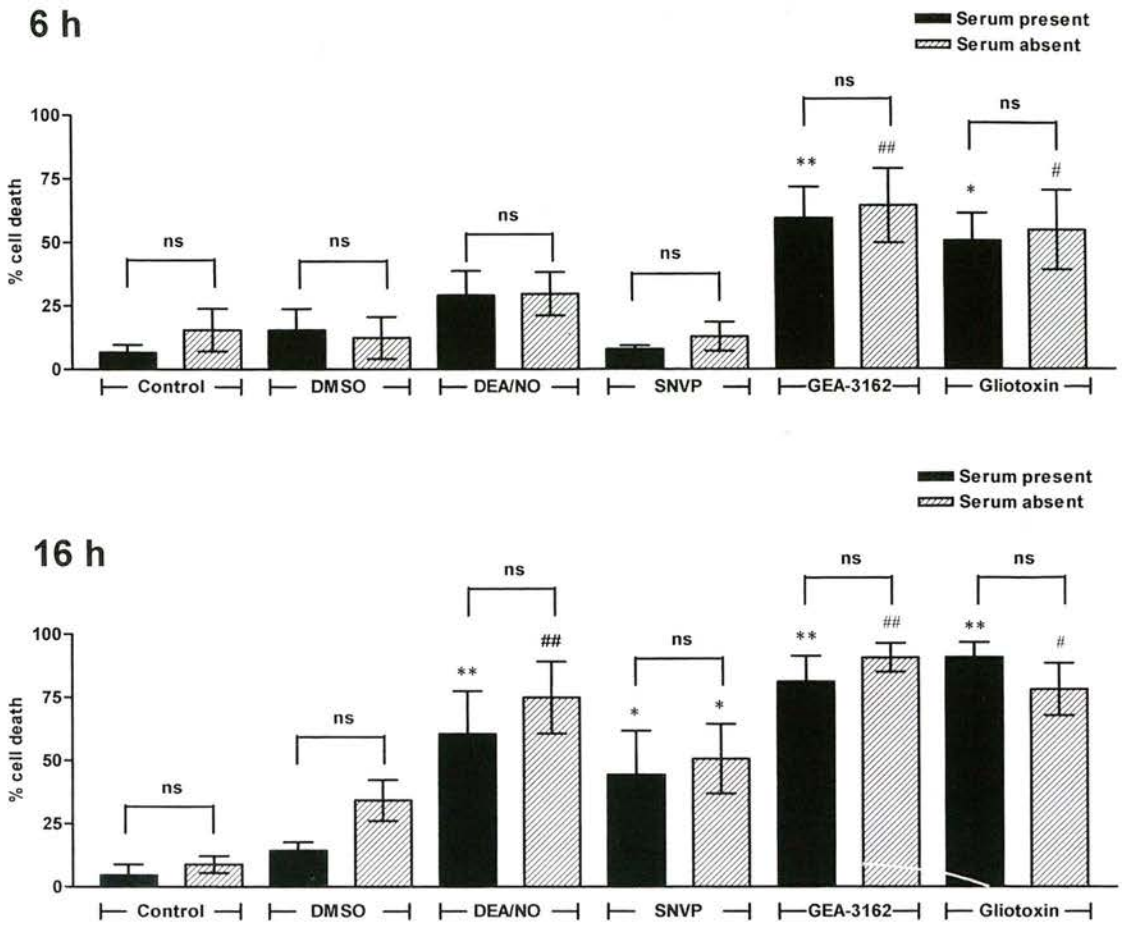

Figure 5.4 The Influence of FCS on Cell Death Induced by NO Donor Compounds in THP-1 Macrophages

Cell death induced by drug vehicle (DMSO; 1%), DEA/NO, SNVP, GEA-3162 (all 100 μM) and gliotoxin (1 $\mu g.ml^{-1}$) was assessed by cell morphology in the presence (solid bars) and absence (striped bars) of heat inactivated foetal calf serum (FCS; 10%) in THP-1 macrophages following treatment for 6 h and 16 h as indicated. Analysis by one-way ANOVA followed by post hoc Tukey test revealed GEA-3162 and gliotoxin induced significant cell death compared to vehicle treated cells both in the presence and absence of serum 6 h and 16 h. DEA/NO and SNVP induced significant cell death compared to vehicle treated cells in the presence and absence of serum at 16 h only. However, the presence or absence of serum did not significantly affect the level of cell death induced by any treatment. * = $P<0.05$, ** = $P< 0.01$ compared to vehicle treated in the presence of serum, and # = $P<0.05$, ## = $P<0.01$ compared to vehicle treated cells in the absence of serum; ns = $P >0.05$; n=6 for all treatments at both time points.

5.3.3.2 Characterisation of Cell Death Induced by NO and NO-Related Species in Human Monocyte-Derived Macrophages

The maximum concentration of drug vehicle (DMSO; 1%) had no effect on annexin V binding (a marker of apoptosis) or PI staining (a marker of necrosis) in either monocytes (figure 5.5), or monocyte-derived macrophages compared to control, untreated cells ($P>0.05$; matched, one-way ANOVA; n=6; figure 5.6). DEA/NO, DETA/NO, SNVP and GSNO (all 100 and 300 μM) did not induce significant annexin V binding or PI staining in either monocytes (figure 5.5), or monocyte-derived macrophages, compared to vehicle treated cells ($P>0.05$; matched, one-way ANOVA; n=6; figure 5.6).

GEA-3162 (100 and 300 μM) and gliotoxin (1 $\mu g.ml^{-1}$) markedly increased the percentage of cells positive for annexin V binding compared to vehicle treated cells in both monocytes (figure 5.5) and monocyte-derived macrophages (figure 5.6). For example, in monocyte-derived macrophages, GEA-3162 (100 μM) increased the percentage of cells positive for annexin V binding from 11.7 ± 4.5% (vehicle treated cells) to 46.8 ± 11.9% ($P<0.01$; matched, one-way ANOVA followed by Dunnett's post hoc test; n=6; figure 5.6). With the exception of the highest concentration of GEA-3126 (300 μM), the increase in annexin V binding occurred in the absence of PI staining (figures 5.5 & 5.6). *P* values quoted refer to statistical analyses performed on results for monocyte-derived macrophages only. Statistical analyses were not carried out on results for monocytes due to low n number (n=3).

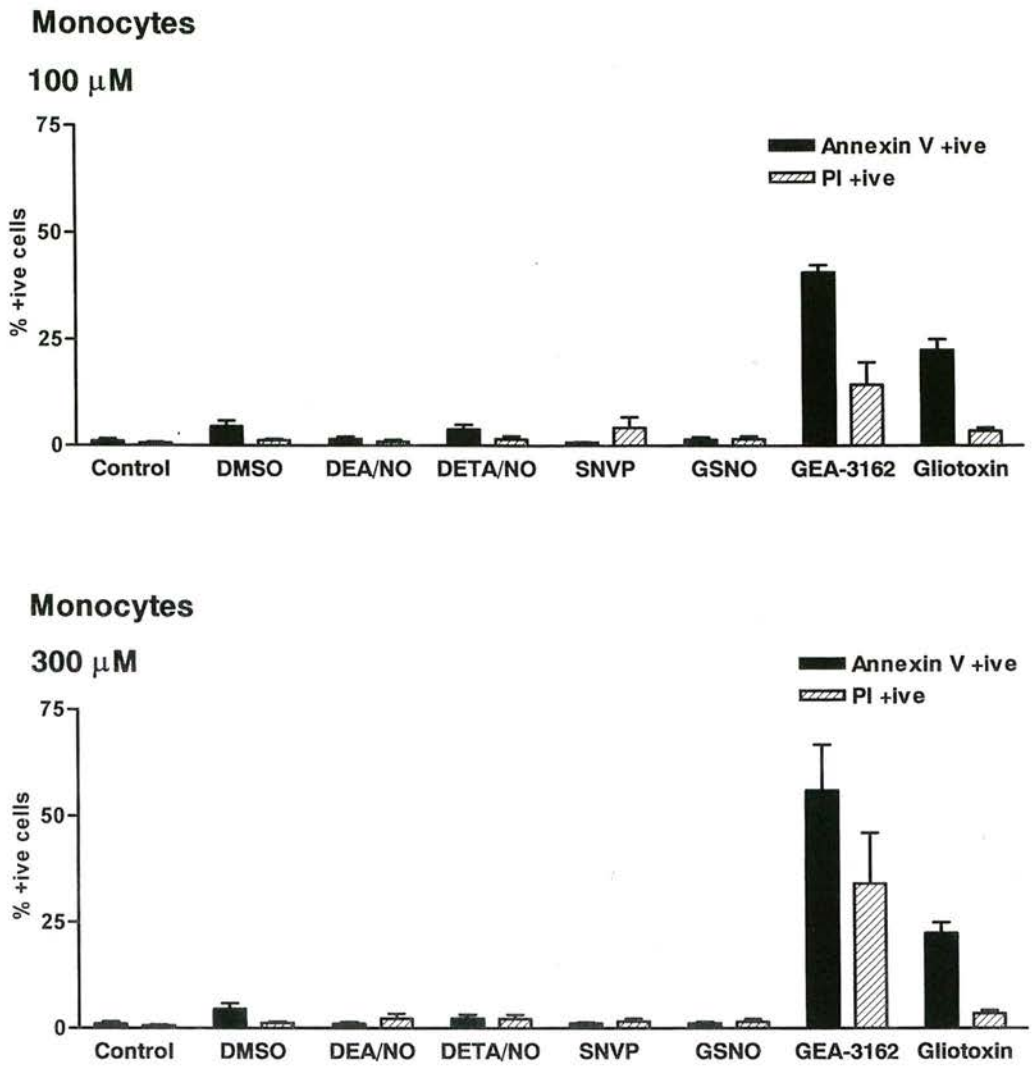

Figure 5.5 Apoptosis and Necrosis Induced by NO and NO-Related Species in Human Monocytes

Cell death induced by NO donor compounds (100 and 300 μM) was characterised as apoptosis or necrosis in human monocytes flow cytometry. The percentage of cells positive for annexin V binding is indicated by solid bars, and for PI staining by striped bars (n=3 for both concentrations). Only GEA-3162 and gliotoxin (1 $\mu g.ml^{-1}$) caused an increase in annexin V binding which occurred in the absence of PI staining except at the higher concentration of GEA-3162 (300 μM).

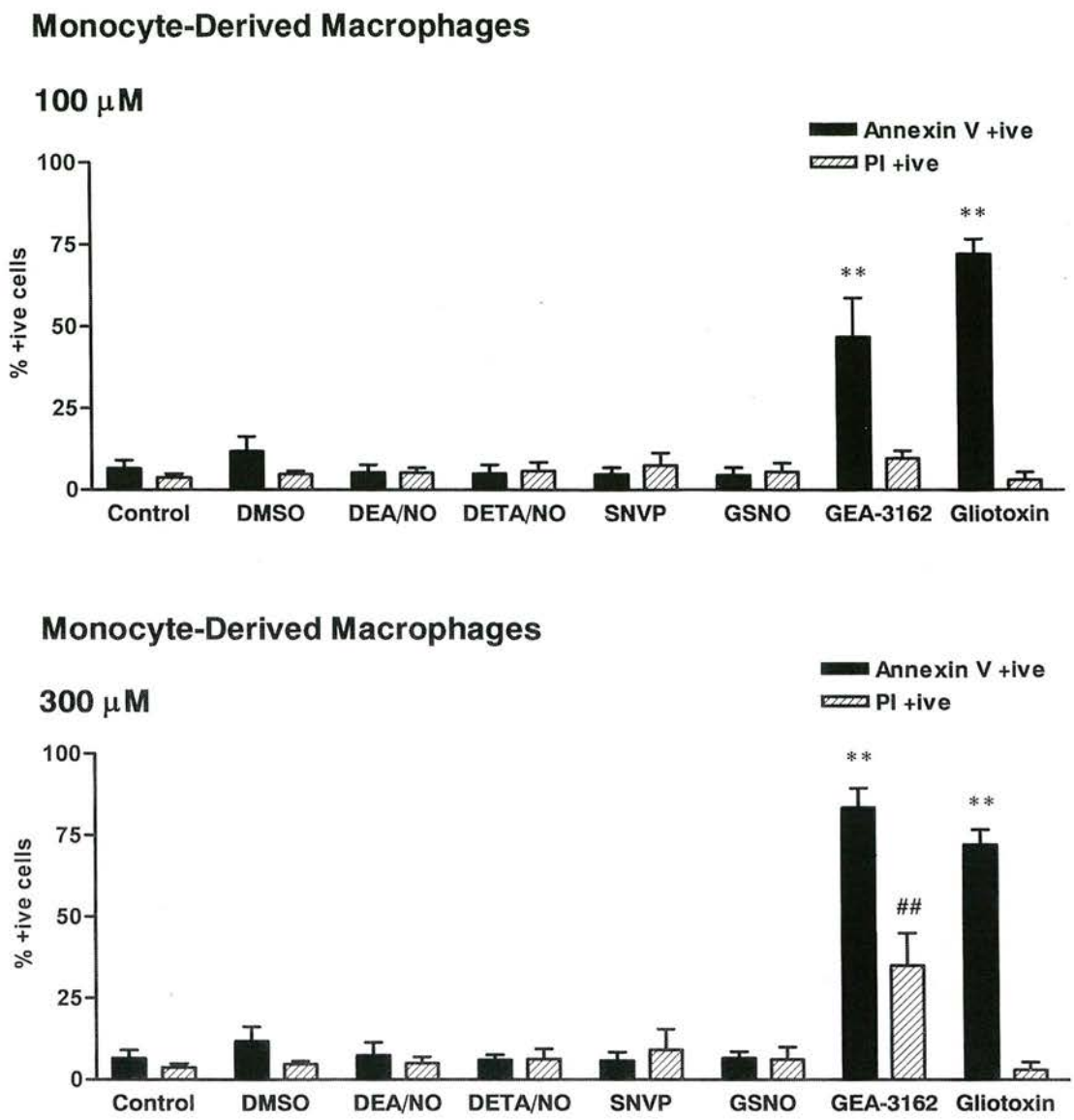

Figure 5.6 Apoptosis and Necrosis Induced by NO and NO-Related Species in Human Monocyte-Derived Macrophages

Cell death induced by NO donor compounds (100 and 300 μM) was characterised as apoptosis or necrosis in human monocyte-derived macrophages by flow cytometry. The percentage of cells positive for annexin V binding is indicated by solid bars, and for PI staining by striped bars. Analysis by matched, one-way ANOVA followed post hoc Dunnett's test revealed GEA-3162 and gliotoxin (1 $\mu g.ml^{-1}$) caused a significant increase in annexin V binding compared to DMSO (1%) treated cells (** = $P<0.01$) which occurred in the absence of PI staining at 100 μM and in combination with significant PI staining at 300 μM (## = $P<0.01$; n=6 for both concentrations).

SOD (500 $U.ml^{-1}$) had no affect annexin V binding or PI staining in monocyte-derived macrophages compared to control (untreated) cells, or vehicle treated cells (data not shown). Similarly, SOD had no further effect on those treatments that did not affect cell viability (diazeniumdiolate and RS-N=O compounds) or on the level of apoptosis induced by gliotoxin (data not shown).

The level of annexin V binding induced by GEA-3162 (10 – 300 μM) was significantly increased by SOD ($P<0.01$; two-way repeated measures ANOVA followed by post hoc Bonferroni's test; n=6; figure 5.7). PI staining remained unaltered by the presence of SOD ($P>0.05$; two-way ANOVA; n=6; figure 5.7).

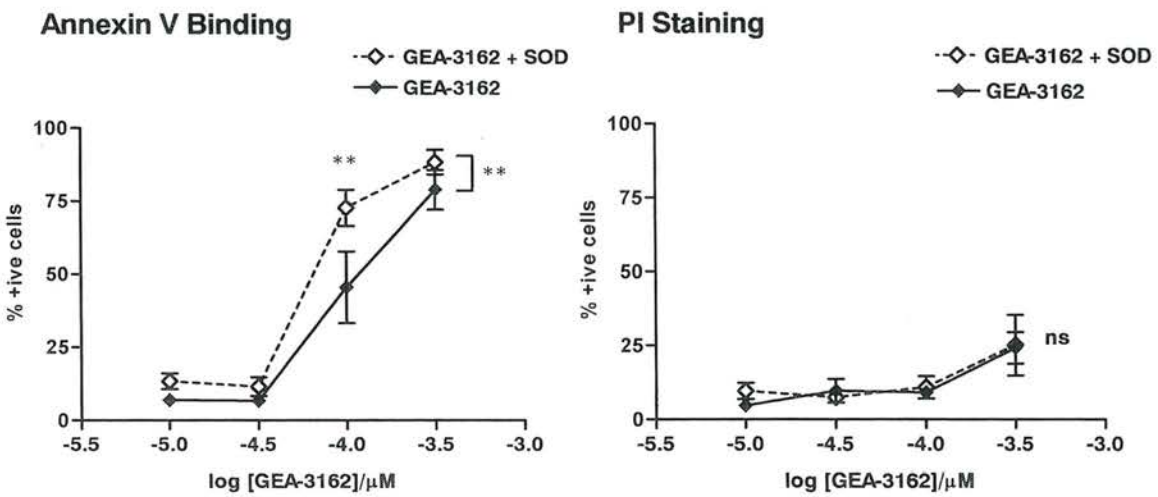

Figure 5.7 The Effect of SOD on GEA-3162-Induced Apoptosis in Human Monocyte-Derived Macrophages

Annexin V binding and PI staining induced by GEA-3162 (10 – 300 μM) was assessed in the absence (solid lines) and presence (dashed lines) of SOD (500 $U.ml^{-1}$) by flow cytometry. Analysis by matched, two-way ANOVA followed by post hoc Bonferroni's test revealed SOD significantly increased the level of annexin V binding, but had no effect on PI staining (** = $P<0.01$; ns = $P>0.05$; n=6).

5.4 Discussion

The results of this study confirm that NO production by LPS-stimulated human macrophages is exceedingly low or absent. Neither the Griess test nor the HPLC method was sensitive enough to detect NOx generated by human macrophages under these conditions. Fluorescence HPLC is capable of detecting NO_2^- in the nM range (Misko et al. 1993; Li et al. 2000; Woitzik et al. 2001; Gharavi and El-Kadi 2003), however, despite this enhanced sensitivity, levels of NO_2^- detected from monocyte-derived macrophages remained below the limit of detection.

Human macrophages have previously been reported to be unable to generate the supraphysiological NO concentrations produced by rodent cells (Albina 1995; Schneemann and Schoedon 2002). However, despite this observed inability to generate NO, iNOS mRNA and protein have been detected in human macrophages isolated from atherosclerotic plaques (Buttery et al. 1996; Luoma et al. 1998; Luoma and Yla-Herttuala 1999; Behr-Roussel et al. 2000). iNOS expression under these circumstances is likely to be an induced response due to the inflammatory nature of atherosclerosis (Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002), and therefore, treatment for 24 h with LPS alone may have been insufficient to cause iNOS up-regulation to levels capable of generating detectable concentrations of NO. Weinberg *et al* reported elevated levels of iNOS mRNA and protein, and of NO_2^- /NO_3^- (measured by Greiss test) in human peritoneal macrophages following LPS stimulation (Weinberg et al. 1995). However, the cells were treated for 4 days, rather than 24 h, and although NO_2^-/NO_3^- generation was augmented by treatment with LPS, this was delivered in combination with interferon-γ (INF-γ), and remained significantly below that produced by murine cells as part of the same study

(Weinberg et al. 1995). A more successful strategy for future attempts to detect NO from human monocyte-derived macrophages may therefore be to stimulate the cells for a longer period of time with LPS in combination with a 'cocktail' of cytokines. A further consideration is NOS substrate or co-factor deficiency. Substrate or co-factor deficient NOS has previously been demonstrated to generate O_2^-, rather than NO (Xia and Zweier 1997; Vasquez-Vivar et al. 1998; Xia et al. 1998; Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1999b; Vasquez-Vivar et al. 2002). However, the medium in which monocyte-derived macrophages are cultured (IMDM) contains L-Arg (0.4 mM; appendix one), so it is perhaps unlikely that NOS would be substrate deficient, unless there was a disorder of Arg transport. Interestingly, Weinberg *et al* reported that although human peritoneal macrophages did not contain the NOS co-factor, tetrahydrobiopterin (BH_4); supplementation with BH_4 did not result in increased NO production, suggesting there may be additional reasons underlying the inability of human macrophages to produce NO (Weinberg et al. 1995).

Despite this apparent inability to generate NO, human macrophages do respond to exogenous NO-related species. Therefore, although the contribution of macrophage-derived NO to processes occurring within the atherosclerotic plaque might be minimal, this cell type remains a realistic target for NO-based therapies in the treatment of atherosclerosis. It is also worth noting that the macrophages used in the current study were isolated from the peripheral blood of healthy volunteers and given the inflammatory nature of atherosclerosis, macrophages within the plaque may possess additional NO generating capacity as a result of inflammation-driven iNOS up-regulation. iNOS up-regulation could potentially have implications for

endothelium-derived NO or NO-based therapies. If macrophage iNOS becomes substrate or co-factor deficient, resulting in O_2^-, rather NO, generation, then any available NO would combine with O_2^-, forming $ONOO^-$ (Saran et al. 1990; Czapski and Goldstein 1995; Goldstein and Czapski 1995; Reiter et al. 2000). This is particularly important because the studies presented here examining the role of NO in macrophage viability, demonstrated that of a range of compounds capable of releasing various NO-related species, only the $ONOO^-$ generator, GEA-3162 is able to induce apoptosis in both human monocytes, and monocyte-derived macrophages. Comparable levels of NOx delivered via RS-N=O (demonstrated by Griess test), or elevated levels of NO radical, delivered either as a rapid and short burst (DEA/NO) or in a prolonged and sustained manner (DETA/NO; chapter three), failed to induce apoptosis in human monocytes and monocyte-derived macrophages.

Several previous reports have demonstrated that NO, delivered by both diazeniumdiolate and RS-N=O compounds, is capable of inducing apoptosis in macrophages (Hibbs et al. 1988; Albina et al. 1993; Sarih et al. 1993; Messmer et al. 1995; Shimaoka et al. 1995; Messmer and Brune 1996b; Messmer and Brune 1996a; Messmer et al. 1996), and recent studies have suggested a central role for S-nitrosylation in macrophage apoptosis (Benhar and Stamler 2005; Hara et al. 2005). However, without exception, these studies have been conducted in rodent cells, either murine macrophage cell lines, such as RAW 264.7 cells, or isolated peritoneal macrophages, and have used a variety of means, including stimulating the L-Arg pathway, NO gas, and various NO donor compounds to deliver NO at relatively high concentrations (usually >mM drug concentration). The concentration of NO, or NO-related species, generated by the NO donor compounds in this study are an order of

magnitude lower than this (in the μM range), indicating that the capacity of NO to induce apoptosis is likely to be concentration-, as well as cell type-, specific, and therefore may not occur *in vivo* as a result of endogenously generated NO.

Initial pilot experiments in THP-1 macrophages, implied that NO radical and RS-N=O, as well as $ONOO^-$, are cytotoxic to human macrophages, but this was not borne out in freshly isolated monocytes or macrophages, which underwent apoptosis in response to $ONOO^-$ only. Interesting, cell death in THP-1 cells in response to DEA/NO did not occur until 16 h, despite NO release from DEA/NO occurring in a rapid and short burst (chapter three). The reasons for this lag time are not clear, but may suggest NO-induced transcriptional up-regulation of proteins involved in the execution of apoptosis, or down-regulation of proteins involved in the inhibition of apoptosis. For example, in murine RAW 264.7 macrophages, NO (delivered by diazeniumdiolate and RS-N=O compounds) causes profound down-regulation of the intracellular caspase inhibitors, inhibitor of apoptosis proteins (IAPs), with inhibition correlating temporally with apoptosis (Manderscheid et al. 2001). The mechanisms of NO-induced cytotoxicity in THP-1 cells has not been examined here, but the observed differences between macrophages of the THP-1 cell line and freshly isolated, terminally differentiated, monocyte-derived macrophages, may be due to the immortalised nature of the THP-1 cells, which could potentially result in altered cell death pathways.

Interestingly, serum starving had no effect on the level of cell death in THP-1 cells, despite being able to both initiate apoptosis, and augment transforming growth factor β (TGF-β)- and hydrogen peroxide (H_2O_2)- induced apoptosis in endothelial cells (Hogg et al. 1999). It is possible that the presence of serum protected

monocytes and monocyte-derived macrophages from NO- or RS-N=O-induced apoptosis. But given that serum withdrawal had no affect on apoptosis in THP-1 cells, macrophages may be less sensitive to serum deprivation than other cell types. Additionally, extrapolation of the relevance of experimental serum starving *in vitro* to the situation *in vivo* must be done with a degree of caution because serum proteins will be present *in vivo*.

It is interesting to note that undifferentiated monocytes undergo apoptosis in response to $ONOO^-$ in the same manner as differentiated monocyte-derived macrophages. Atherosclerotic plaques are dynamic and cells are constantly being recruited to the core. Therefore, $ONOO^-$ appears able to induce apoptosis in those cells newly recruited to the site of inflammation before differentiation, and in those cells that are already resident in the core and have undergone differentiation to macrophages.

Apoptosis was characterised in human monocytes and monocyte-derived macrophages by annexin V binding in the absence of propidium iodide (PI) staining. This pattern indicates cell surface phosphatidylserine (PS) exposure with the outer membrane remaining intact and, therefore, confirms that cell death is occurring by apoptosis, rather than necrosis. Although higher concentrations of $ONOO^-$ appear to cause a degree of PI staining, indicating some necrosis in addition apoptosis, PI staining may not necessarily indicate primary necrosis in this case. This is because the cells were incubated in the presence of $ONOO^-$ for 24 h, whilst the release profile of GEA-3162 was demonstrated in earlier studies (chapter three) to be in the region of approximately 4 h in IMDM. Therefore, it is possible that apoptosis is initiated during the early stages of treatment, and in the absence of a population of

phagocytes, the apoptotic cells enter late apoptosis or secondary necrosis, resulting in membrane rupture and allowing PI staining. In order to clarify this, apoptosis should be confirmed by alternative methods, such as DNA laddering or deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) staining; constraints of time did not permit such experiments to be carried out as part of the current study.

$ONOO^-$, at comparable concentrations to those used here, has previously been shown to induce apoptosis in several cell types, including HL-60 (a human leukaemia cell line), freshly isolated rat thymocytes, and murine macrophages (Lin et al. 1995; Salgo et al. 1995; Sandoval et al. 1997a; Sandoval et al. 1997b), but this is first report of $ONOO^-$ inducing apoptosis in human monocytes/macrophages.

Mechanisms for the disparate induction of apoptosis by $ONOO^-$ compared NO and RS-N=O in freshly isolated monocytes and monocyte-derived macrophages have not been investigated here. One possibility is divergent effects on nuclear transcription factor-κB (NF-κB). NF-κB transcriptional activity leads to the expression of many anti-apoptotic proteins, including IAPs and the Bcl-2 family of proteins, that limit caspase activity (Delhalle et al. 2004). The pro-apoptotic agent, gliotoxin, used as a positive control in these studies, induces apoptosis by inhibiting NF-κB activation {Pahl, 1996 #689;Ward, 1999 #91}, thereby preventing the synthesis of survival proteins. Therefore, $ONOO^-$ may induce apoptosis in human monocytes and monocyte-derived macrophages by inhibiting NF-κB activation, whilst NO may sustain NF-κB activity in order to prevent apoptosis. $ONOO^-$ has been shown to inhibit NF-κB activity in cardiomyocytes and endothelial cells (Levrand et al. 2005), whilst NO sustains its activity in chondrocytes and RAW

274.7 macrophages (von Knethen et al. 1999; Clancy et al. 2004). However, the role of both $ONOO^-$ and NO in NF-κB inhibition/activation remains controversial, and in fact, the opposite actions for these molecules have been also described, with NO suppressing, and $ONOO^-$ sustaining, NF-κB activity in VSMCs and endothelial cells (Cooke and Davidge 2002; Hattori et al. 2004). The actions of $ONOO^-$ and NO on NF-κB activity are clearly complex and cell-type specific. In addition to actions on NF-κB transcription, $ONOO^-$ is also known to be able to initiate DNA strand breaks during $ONOO^-$-mediated cytotoxicity of murine macrophages (Szabo et al. 1996; Zingarelli et al. 1996; Guidarelli et al. 2000). Therefore, whilst divergent actions on NF-κB is one potential mechanism to rationalise variations in the NO-related species induction of apoptosis, it is by no means the only possibility and thorough investigation in this particular cell type is required before the precise mechanism of $ONOO^-$-induced apoptosis can be identified.

The downstream effectors of $ONOO^-$ -induced apoptosis also remain to be fully elucidated. $ONOO^-$ -induced apoptosis in rat thymocytes is inhibited by the anti-oxidant, Trolox, indicating that apoptosis is triggered by oxidising species (Salgo et al. 1995). If this were the case in human monocyte-derived macrophages, SOD might be expected to inhibit apoptosis. In the current study, rather than inhibiting $ONOO^-$ -induced apoptosis, SOD augmented the level of apoptosis. There are two possible explanations for this; firstly, earlier EPR experiments (chapter three) demonstrated that the concentration of SOD used in these studies (500 $U.ml^{-1}$) reduces the concentration of oxidising species generated by GEA-3162 by approximately half. Therefore, as this concentration of SOD is insufficient to totally abolish the production of oxidising species, there may be sufficient residual oxidising

species remaining to trigger apoptosis. Alternatively, O_2^- may not be the mediator of apoptosis. SOD catalyses the conversion of O_2^- to H_2O_2, prior to subsequent catalytic conversion of H_2O_2 to H_2O and O_2 by the enzyme catalase (Beckman and Koppenol 1996). H_2O_2 has been shown to induce apoptosis in murine cells by inhibiting caspase enzymes (Borutaite and Brown 2001; Borutaite and Brown 2003). Therefore, H_2O_2 may be the apoptotic trigger. This could be further investigating in human monocyte-derived macrophages by examining whether a combination of SOD and catalase successfully abolishes $ONOO^-$ - induced apoptosis.

It would be of interest to investigate if $ONOO^-$ - induced apoptosis in human monocyte-derived macrophages occurs via the mitochondrial (or stress) pathway, or by the death receptor pathway. Cytochrome-*c* release and mitochondrial depolarisation have been reported to precede NO-induced apoptosis in a variety of cells, including in murine macrophages (Brown and Borutaite 1999; Hortelano et al. 1999; Bal-Price and Brown 2000; Brown and Borutaite 2001; Brown and Borutaite 2002; Maneiro et al. 2005). Therefore, it is reasonable to speculate that $ONOO^-$ - induced apoptosis in human macrophages might trigger mitochondrial-dependent apoptosis. However, given the specificity and highly regulated nature of apoptosis this would require through investigation in this cell type before the specific pathway is identified.

In summary, the salient finding of these studies is that of a range of compounds capable of generating NO or NO-related species, only the $ONOO^-$ generator is capable of inducing apoptosis in human monocytes and monocyte-derived macrophages. This NO-species variation could potentially be exploited as a means to achieve cell-selective induction of apoptosis in the inflammatory cell

population of atherosclerotic plaques, with the aim of stabilising the plaque by limiting disease progression, or aiding plaque regression. However, this therapeutic strategy can only be considered after through investigation into the effects of $ONOO^-$ on other cell types critical to plaque stability.

Chapter Six

The Role of the NO:cGMP Pathway in Protecting Human Monocyte-Derived Macrophages and Vascular Smooth Muscle Cells Against Cell Death

6. The Role of the NO:cGMP Pathway in Protecting Human Monocyte-Derived Macrophages and Vascular Smooth Muscles Cells Against Cell Death

6.1 Introduction

NO can be both pro- and anti-apoptotic, depending on the nature and local concentration of the NO-related species generated in the microenvironment, as well as the characteristics of the target cell. Current evidence suggests that lower concentrations of eNOS- and nNOS-derived NO are cytoprotective via primarily cGMP-dependent mechanisms, whilst higher, supraphysiological concentrations generated in some cell types by iNOS under pathological conditions, mediate apoptosis via mechanisms independent of cGMP signalling (Nicotera et al. 1997). Apoptosis, and subsequent phagocytic removal of apoptotic cells, is now thought to be crucial to the successful resolution of the inflammatory response (Haslett 1997; Maderna and Godson 2003; Taylor et al. 2003; Rossi et al. 2004).

Disparities between the sensitivity of different cell types to NO-induced cytoxocity suggests the presence of protective mechanism(s) in those cell types resistant to NO-evoked cell death. This protective mechanism may depend on the anti-apoptotic qualities of NO itself. Non-toxic doses of NO, and agents that elevate cGMP, have been demonstrated to protect rodent macrophages and VSMCs against subsequent NO-induced cell death (von Knethen et al. 1999; Yoshioka et al. 2003; Pan et al. 2004).

In healthy blood vessels, the endothelium secretes eNOS-derived NO to control vasomotor tone and to maintain the integrity of the vessel wall. The ability of low concentrations of NO to protect cells from a cytotoxic insult may well be an additional cardio-protective property of NO. During atherogenesis the endothelium becomes dysfunctional, resulting in decreased NO production, hence removing this cytoprotection. However, rather than contributing directly to the atherogenic process, this may, in fact, be the first line of defence in an attempt to resolve the vascular inflammation now generally accepted to underlie atherosclerosis (Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002). Allowing apoptosis, and subsequent phagocytosis, to proceed by removing the protective actions of NO, might prevent the accumulation of the necrotic inflammatory cells in the sub-endothelial space that characterises atherosclerosis. However, if the population of phagocytes is overwhelmed by the magnitude and duration of the inflammatory response, then apoptotic cells remaining *in situ* would eventually become necrotic, resulting in cell membrane rupture, release of the pro-inflammatory mediators contained within the cell, ultimately causing an exacerbation of the inflammatory response. Furthermore, the removal of NO-mediated cytoprotection from VSMCs might contribute to weakening of the plaque cap, triggering plaque rupture, and ultimately leading to the acute clinical consequences of atherosclerosis (Bauriedel et al. 1999; Leskinen et al. 2003). Thus, the decrease in eNOS-derived NO may be part of the initial defence mechanism that goes awry and develops from being an initial protective response to vascular inflammation, to eventually contributing to the disease process itself.

The mechanism of such 'priming' by NO is currently unclear, and has been reported to be cGMP-dependent (Yoshioka et al. 2003). Alternatively, NO might cause nuclear transcription factor κB (NF-κB) activation, leading to subsequent cyclooxygenase 2 (COX-2) up-regulation, although the mechanism by which COX-2 protects cells from apoptosis remains to be elucidated (von Knethen et al. 1999).

Development of inhibitors of the sGC:cGMP pathway including the specific sGC inhibitor, ODQ (Garthwaite et al. 1995; Schrammel et al. 1996), and phosphodiesterase (PDE) inhibitor, IBMX (van Staveren et al. 2001), together with the recent advent of NO-independent stimulators of sGC such as YC-1 and BAY 41-2772 (Ko et al. 1994; Stasch et al. 2001), allow the role of cGMP in the protective effects of NO to be examined in detail. YC-1 and BAY 41-2772 both have beneficial effects in the cardiovascular system including anti-platelet effects, inhibition of VSMC proliferation and anti-hypertensive properties (Ko et al. 1994; Stasch et al. 2001; Tulis et al. 2002). Furthermore, YC-1 has recently been demonstrated to prevent NO-mediated apoptosis in VSMC, suggesting that cGMP might confer the anti-apoptotic effects of NO (Pan et al. 2004).

Previous studies demonstrating the cyoprotective action of pre-treatment with low concentrations of NO have used a variety of NO donor compounds (sodium nitroprusside; SNP, and GSNO) at relatively high concentrations (>1mM drug concentration; von Knethen et al. 1999; Yoshioka et al. 2003), which are likely to generate NO at higher concentrations than the normal NO producing capacity of the healthy endothelium. Concentrations of these compounds ~100 fold lower than those used in the studies quoted above are known to have considerable anti-platelet and vasodilator actions (Sogo et al. 2000; Miller et al. 2004).

Therefore, the aims of these studies were to rationalize the various paradoxes relating to the disparate ability of NO and NO-related species to induce apoptosis. In order to investigate this, the hypothesis that the anti-apoptotic actions of NO are due to physiologically relevant concentrations of NO and ensuing increases in cGMP, whilst the pro-apoptotic actions of NO are due to higher, supraphysiological doses of NO and NO-related species was tested in human monocyte-derived macrophages and BAoSMC. This was examined by stimulating the NO:cGMP pathway with a more realistically physiological dose of NO in combination with augmentation of cGMP levels, prior to subsequent treatment with a concentration of $ONOO^-$ known to induce apoptosis. Additionally, the hypothesis that cellular protection is mediated by cGMP, rather than NO radical *per se* was tested by treating the cells with NO in combination with sGC inhibition.

6.2 Methods

6.2.1 Functional Studies In Isolated Rat Aortae

In order to test the efficacy of DETA/NO in combination with the NO-independent sGC stimulator, BAY 41-2772, and the non-specific phosphodiesterase (PDE) inhibitor, IBMX, relaxation in response to DETA/NO in the absence and presence of supramaximal BAY 41-2772 (1μM) and IBMX (1μM) was measured as isometric tension in isolated rat thoracic aortic rings as described chapter two (section 2.7). Relaxations are expressed as a percentage of the maximum contraction in response to EC_{80} phenylephrine (PE). Following pre-contraction with EC_{80} PE (0.1 μM), cumulative concentration-responses to DETA/NO (0.001 – 100 μM) were obtained in each aortic ring in order to establish the concentration of DETA/NO

giving approximately 75% relaxation (EC_{75}). The EC_{75} DETA/NO was selected for subsequent experiments with cGMP modulators so that it would be possible to observe additional responses evoked by BAY 41-2772 and IBMX.

Following pre-contraction with EC_{80} PE, the relaxation in response to EC_{75} DETA/NO (10 μM) in the absence and presence of BAY 41-2772 (1μM) or IBMX (1μM) was measured in each aortic ring. BAY 41-2772 was used in combination with DETA/NO because recent evidence has suggested that rather than direct stimulation sGC, the actions of this compound are a result of the synergistic effects of inhibition of PDE V coupled with sensitisation of sGC toward endogenous NO (Friebe et al. 1998; Mullershausen et al. 2004).

6.2.2 Cell Culture

6.2.2.1 Isolation and Culture of Human Monocyte-Derived Macrophages

Human monocytes were isolated from peripheral blood of healthy volunteers as described in chapter two (section 2.2.2) and cultured (37 °C; 95% O_2: 5% CO_2) in IMDM (containing supplements plus 10% autologous serum) for 5-7 days to allow differentiation into monocyte-derived macrophages (section 2.2.2.1). At the end of this period, the IMDM was aspirated from the wells and replaced with fresh IMDM containing experimental treatments (see section 6.2.3 below).

6.2.2.2 Bovine Aortic Smooth Muscle Cell Culture

Bovine aortic smooth muscle cells (BAoSMC) were cultured in DMEM (plus supplements) as described in chapter two (section 2.2.3). Cells were seeded in 48-well, flat-bottomed tissue culture plates and cultured (37 °C; 95% O_2: 5% CO_2) for

24 h prior to experimental protocol in order to allow cells to adhere to the plate. At the end of this period, the DMEM was aspirated from the wells and replaced with fresh DMEM plus experimental treatments (see section 6.2.3 below)

6.2.3 Experimental Protocol For Cell Culture Experiments

Following the initial culture period as appropriate for each cell type, cells were treated (24 h; 37°C: 95% O_2; 5% CO_2) with the following pre-treatments: no treatment (control); low concentration NO (DETA/NO; 10 μM); low concentration NO (DETA/NO; 10 μM) + BAY 41-2272 (1 μM) to augment cGMP production; and low concentration NO (DETA/NO; 10 μM) + IBMX (1 μM) to prevent cGMP degradation. In order to establish that any protection conferred on the cells is due to cGMP, rather than NO *per se*, a group was treated with DETA/NO (300 μM) in the presence of the specific sGC inhibitor, ODQ (20 μM). Following incubation for 24 h in the presence of these pre-treatments, the medium was aspirated from the wells and the cells washed twice in Dulbeccos's phosphate buffered saline (PBS; containing $CaCl_2 \cdot 6H_2O$; 0.133 M and $MgCl_2 \cdot 6H_2O$; 0.1 M) prior to addition of fresh full media. Cells were then incubated for a further 24 h (37°C: 95% O_2; 5% CO_2) in the absence and presence of the $ONOO^-$ generator, GEA-3162 (100 μM), or the apoptotic agent, gliotoxin (1 $\mu g.ml^{-1}$; figure 6.1). This concentration of GEA-3162 was selected as it had previously been demonstrated to induce significant apoptosis in human monocyte-derived macrophages without causing necrosis (chapter five), and to induce necrosis in BAoSMC (chapter four). Following treatment with GEA-3162 or

gliotoxin, cell death was assessed by flow cytometry as described in chapter two (section2.3.3).

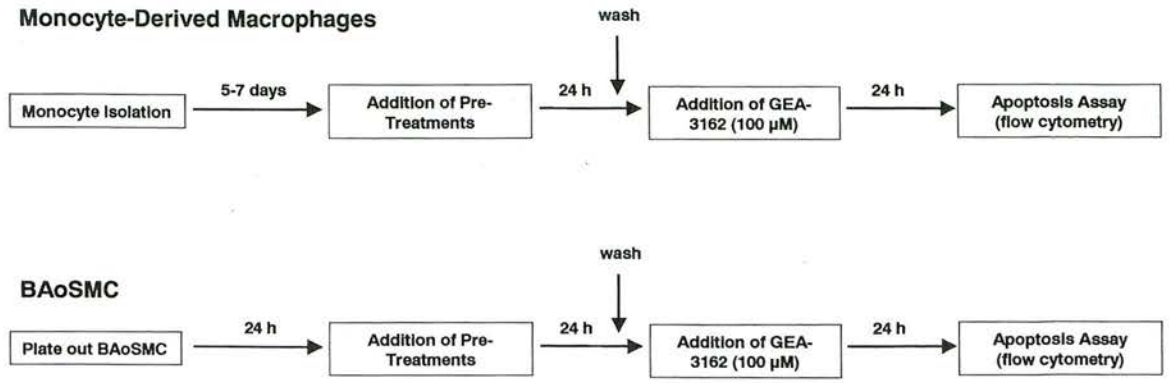

Figure 6.1 Schematic Representation of Experimental Protocol for Human Monocyte-Derived Macrophages and BAoSMC

6.2.4 Measurement of cGMP

Cells were incubated (37°C: 95% O_2; 5% CO_2) for 24 h in the presence of the pre-treatments described above plus IBMX (250 µM). Addition of IBMX (250 µM) was necessary to inhibit PDEs, and therefore prevent cGMP breakdown. At the end of this 24 h period, cells were lysed (Triton X 100; 2%) and cGMP measured in human monocyte-derived macrophage and BAoSMC lysates as described in chapter two (section 2.5).

6.2.5 Measurement of Protein

To avoid discrepancies in the exact number of cells seeded in each well, a protein assay was carried out in order to express the concentration of cGMP per mg of protein. Although the cells are counted and diluted to equal concentration prior to

seeding (chapter two; sections 2.2.2.1 & 2.2.3), the method used is fairly crude and may not be completely consistent, resulting in variations in the number of cells seeded in each well. The greater the density of cells, the greater the cGMP producing capacity of the cells in any given well will be. Expressing the concentration of cGMP per mg of protein allows for such variation in cell density.

The total concentration of protein present in each well was measured by bicinchoninic acid (BCA) colourimetric assay as described in chapter two (section 2.6).

6.3 Results

6.3.1 Functional Studies in Isolated Rat Aortae

DETA/NO (0.001 – 100 μM) evoked concentration-dependent relaxation in isolated aortic rings (figure 6.2). For subsequent experiments, a concentration of 10 μM was selected as approximately EC_{75}.

BAY 41-2772 (1 μM) and IBMX (1 μM) both induced relaxation in the absence of DETA/NO (43.5 ± 18.5% for BAY 41-2772 and 39.5 ± 3.2% for IBMX). Subsequent addition of DETA/NO (10 μM) induced a more profound relaxation than DETA/NO alone (89.9% *vs* 72.3% for BAY 41-2772 the in presence of DETA/NO compared to DETA/NO alone and 79.5% *vs* 58.5% for IBMX the in presence of DETA/NO compared to DETA/NO alone).

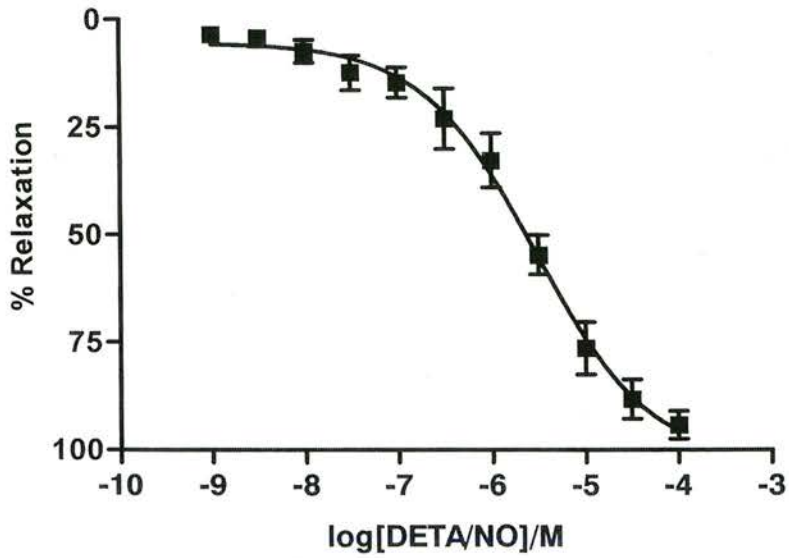

Figure 6.2 Cumulative Concentration-Response Curve for DETA/NO

Relaxation in response to DETA/NO (0.001 – 100 μM) was measured as isometric tension in isolated rat aortic rings following pre-contraction with EC_{80} PE (0.1 μM). Data are expressed as mean ± SEM. (n=4).

6.3.2 Effect of Low Concentration NO Pre-treatment on $ONOO^-$ -Induced Cell Death

6.3.2.1 Human Monocyte-Derived Macrophages

DETA/NO (10 μM; as selected from vasodilator experiments) in the absence and presence of BAY 41-2772 (1 μM) or IBMX (1 μM), and DETA/NO (300 μM) in presence of ODQ (20 μM), did not cause increased levels of either annexin V binding, or PI staining compared to control, untreated cells (P>0.05 for all treatments; matched, one-way ANOVA; n=8; figure 6.3).

In common with previous experiments (chapter five), GEA-3162 (100 μM) induced a significant increase in the percentage of cells positive for annexin V binding without significantly affecting PI staining compared to control cells (20.1 ± 5.3% *vs* 51.4 ± 5.9% for annexin V binding; P<0.001; matched, one-way ANOVA followed by post hoc Tukey test; n=8, and 16.6 ± 7.2% *vs* 11.5 ± 3.4% for PI staining; P>0.05; matched, one-way ANOVA; n=8; figure 6.3). The level of annexin V binding induced by GEA-3162 was significantly attenuated in those cells that had been subjected to previous treatment with DETA/NO (10 μM) in combination with BAY 41-2772 (51.4 ± 5.9% *vs* 31.8 ± 3.6%; P<0.01) or IBMX (51.4 ± 5.9% *vs* 30.4 ± 6.6%; P<0.01; matched, one-way ANOVA followed by post hoc Tukey test; n=8; figure 6.3). Pre-treatment with DETA/NO (10 μM) alone, or DETA/NO (300 μM) in combination with ODQ was unable to cause a significant reduction in GEA-3162-induced annexin V binding (P>0.05; matched, one-way ANOVA; n=8; figure 6.3).

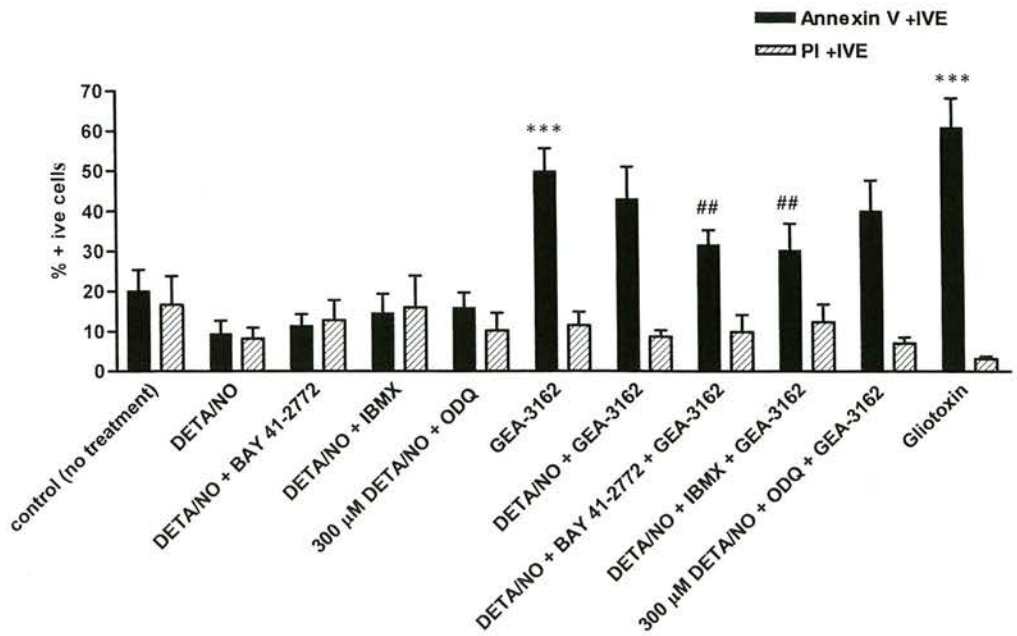

Figure 6.3 The Effect of Pre-treatment with DETA/NO in Combination with Bay 41-2772 and IBMX on $ONOO^-$ -Induced Apoptosis In Human Monocyte-Derived Macrophages

Cell death in human monocyte-derived macrophages was assessed by flow cytometry following pre-treatment (24 h) of cells with DETA/NO (10 µM) in combination with BAY 41-2772 (1 µM) and IBMX (1 µM) or DETA/NO (300 µM) in presence of ODQ (20 µM), followed by further treatment (24 h) with GEA-3162 (100 µM). Additionally, a set of cells was treated for the final 24 h with gliotoxin (1 $\mu g.ml^{-1}$). The percentage of cells positive for annexin V binding is indicated by solid bars, and for PI staining by striped bars. Analysis by matched, one-way ANOVA followed by post hoc Tukey test revealed GEA-3162 and gliotoxin induced significant annexin V binding in the absence of PI staining compared to control, untreated cells (*** = $P<0.001$). The GEA-3162-induced increase in annexin V binding was significantly attenuated by pre-treatment of the cells with DETA/NO (10 µM) in the presence of BAY 41-2772 or IBMX (## = $P<0.01$ compared to GEA-3162 treated cells; n=8).

6.3.2.2 Bovine Aortic Smooth Muscle Cells

DETA/NO (10 μM) in the absence and presence of BAY 41-2772 (1 μM) or IBMX (1 μM), and DETA/NO (300 μM) in presence of ODQ (20 μM), did not cause increased levels of either annexin V binding, or PI staining compared to control, untreated cells ($P>0.05$ for all treatments; unpaired, one-way ANOVA; n=6; figure 6.4).

GEA-3162 (100 μM) induced a significant increase in the percentage of cells positive for annexin V binding compared to control, untreated cells (70.0 ± 5.7% *vs* 38.8 ± 9.9%; $P<0.001$; unpaired, one-way ANOVA followed by post hoc Tukey test; n=6; figure 6.4). However, this occurred in combination with a significant increase in the percentage of cells positive for PI staining (52.1 ± 10.9% *vs* 20.1 ± 3.9%; $P<0.001$; unpaired, one-way ANOVA followed by post hoc Tukey test; n=6; figure 6.4). This pattern was repeated in cells treated with the apoptotic agent, gliotoxin (1 $\mu g.ml^{-1}$) where annexin V binding and PI staining were both significantly increased above control (67.5 ± 3.9% *vs* 38.8 ± 9.9%; $P<0.001$ for annexin V binding and 51.1 ± 3.6% *vs* 20.1 ± 3.9%; $P<0.001$ for PI staining; unpaired, one-way ANOVA followed by post hoc Tukey test; n=6; figure 6.4).

Pre-treatment (24 h) with DETA/NO (10 μM) in the absence and presence of BAY 41-2772 or IBMX, or DETA/NO (300 μM) in the presence of ODQ, followed by subsequent treatment (24 h) with GEA-3162 did not reduce the level of either annexin V binding or PI staining compared to treatment with GEA-3162 in isolation (figure 6.4). Cell death in cells pre-treated as described above remained significantly above that in control, untreated cells.

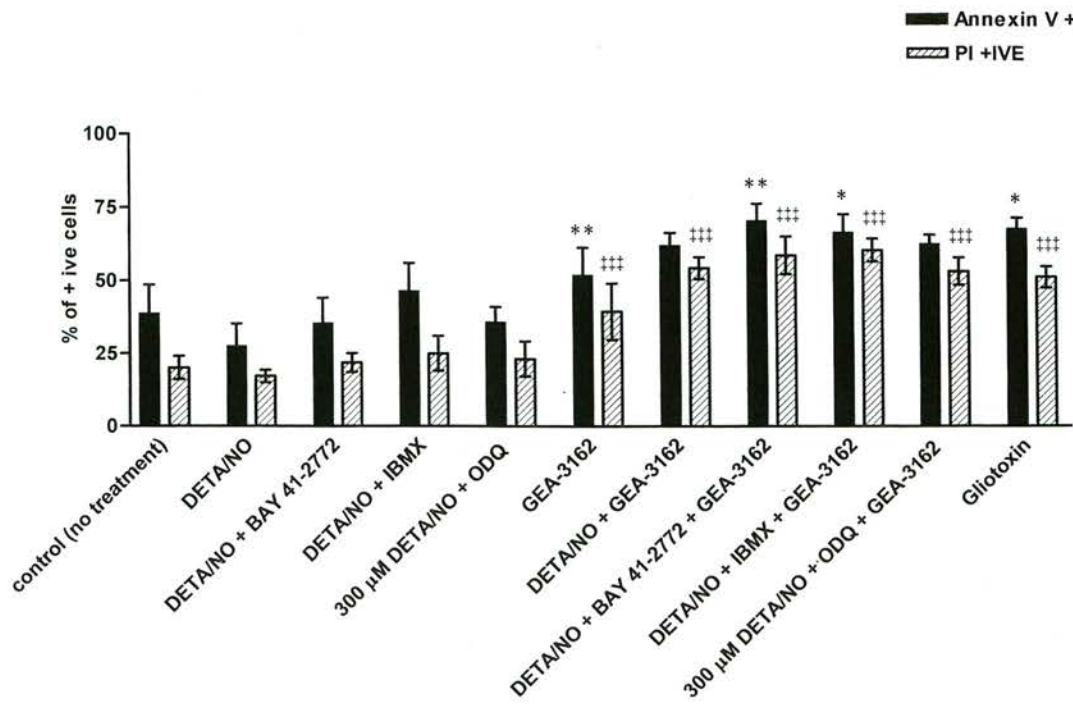

Figure 6.4 The Effect of Pre-treatment with DETA/NO in Combination with Bay 41-2772 and IBMX on $ONOO^-$ -Induced Apoptosis In Bovine Aortic Smooth Muscle Cells

Cell death in BAoSMC was assessed by flow cytometry following pre-treatment (24 h) of cells with DETA/NO (10 µM) in combination with BAY 41-2772 (1 µM) and IBMX (1 µM) or DETA/NO (300 µM) in presence of ODQ (20 µM), followed by further treatment (24 h) with GEA-3162 (100 µM). Additionally, a set of cells was treated for the final 24 h with gliotoxin (1 $\mu g.ml^{-1}$). The percentage of cells positive for annexin V binding is indicated by solid bars, and for PI staining by striped bars. Analysis by unpaired, one-way ANOVA followed by post hoc Tukey test revealed gliotoxin and all treatments involving GEA-3162 induced significant annexin V binding in combination with PI staining compared to control, untreated cells (* = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$ for annexin V binding and ‡‡‡ = $P<0.001$ for PI staining). Pre-treatment of the cells with DETA/NO (10 µM) in the presence of BAY 41-2772 or IBMX and DETA/NO (300 µM) in the presence of ODQ had no effect on the level of annexin V binding or PI staining induced by GEA-3162 ($P>0.05$; n=6)

6.3.3 cGMP and Protein Measurements

6.3.3.1 Human Monocyte-Derived Macrophages

Despite use of the acetylated procedure, specifically designed to detect low concentrations of cGMP, the majority of the pre-treatments described above generated levels of cGMP in human monocyte-derive macrophages below the limit of detection for the assay (0.08 $pM.ml^{-1}$). The only exception to this was in those cells treated with DETA/NO (10 μM) in the presence of BAY 41-2772 (1 μM) in four out of a total of six experiments. When compared to the corresponding protein concentration, this treatment resulted in an average of 2.8 pM cGMP per mg of protein (n=4). The average concentration of protein per well was 0.35 mg.

6.3.3.2 Bovine Aortic Smooth Muscle Cells

None of the treatments resulted in detectable levels of cGMP in BAoSMC. The average concentration of protein per well was 0.16 mg.

6.4 Discussion

The results from these studies demonstrate that pre-treatment with agents that elevate cGMP levels protect monocyte-derived macrophages against apoptosis, but not BAoSMCs against necrosis. $ONOO^-$ -induced apoptosis was attenuated in human monocyte-derived macrophages following prior incubation with DETA/NO in combination with BAY 41-2772 to augment cGMP production, or with IBMX to inhibit cGMP breakdown. However, these treatments failed to abrogate $ONOO^-$ -induced necrosis in BAoSMC.

These findings, together with the observation that a higher concentration of DETA/NO in combination with the specific sGC inhibitor, ODQ, failed to protect

monocyte-derived macrophages against apoptosis, suggest that such protection is conferred by cGMP. cGMP has previously been demonstrated to be responsible for the anti-apoptotic actions of NO in macrophages (Heinloth et al. 2002; Yoshioka et al. 2003). Although it was not possible to measure cGMP concentrations in these studies, previous studies (Stasch et al. 2001) and pilot experiments in rat aortae demonstrated that the doses of DETA/NO, BAY 41-2772 and IBMX used in these experiments are biologically active. The inability to detect cGMP is therefore likely due to the density of cells seeded in each well being too low to provide a cell population with sufficient overall cGMP-producing capacity in the range of detection of the assay. Additionally, measuring cGMP at the end of the 24 h pre-incubation period may not be the best time point. cGMP production would be predicted to begin immediately upon exposure to NO, and therefore, may have peaked and declined earlier in the 24 h period, despite the presence of the PDE inhibitor, IBMX. However, because the cells are lysed in order to measure intracellular cGMP concentration, it is not possible to examine cGMP levels sequentially at timed intervals in the same set of cells over the 24 h incubation period.

It is interesting that although cGMP might be predicted to be elevated early during the initial 24 h incubation, cellular protection persists for a further 24 h period after this. There are two possibilities to explain this: firstly, BAY 41-2772 and IBMX might persist in the cells despite the washing of wells with PBS and the change of medium after the initial 24 h pre-treatment period. Secondly, cGMP activates downstream transcriptional events in order to confer protection against apoptosis. Apoptosis normally proceeds due to regulation of various pro- and anti-apoptotic factors within the cell, such as the Bcl-2 family of proteins, cytochrome-*c*

release from the mitochondria, and caspase signalling (Alnemri 1997; Kluck et al. 1997; Miller 1997; Nicholson and Thornberry 1997; Thornberry 1997; Yang et al. 1997; Green and Reed 1998; Zimmermann et al. 2001). In order to prevent apoptosis, cGMP is likely to regulate one or more of these processes. Possibilities include, for example, inhibiting apoptosis by phosphorylation of pro-apoptotic proteins, or by regulating gene expression of anti-apoptotic proteins, such as anti-apoptotic members of the Bcl-2 family, or the inhibitor of apoptosis proteins (AIPs), which are inhibitors of the pro-apoptotic caspase enzymes (Liston et al. 1996; Roy et al. 1997; Deveraux and Reed 1999; Deveraux et al. 1999).

The downstream effectors of cGMP protection have not been investigated in this study, but previous studies have suggested that cGMP activates protein kinase G (PKG) during NO-dependent cytoprotection (Yoshioka et al. 2003). PKG may alter the balance of pro- and anti-apoptotic factors within the cell in favour of the later. For example, PKG has been demonstrated to prevent hydrogen peroxide (H_2O_2)-induced apoptosis in cultured astrocytes by inhibiting the opening of the mitochondrial transition pore, and consequent pro-apoptotic cytochrome *c* release from the mitochondria (Takuma et al. 2001). This inhibition had a rapid onset (within 30 min) and although prevention of loss of mitochondrial membrane potential persisted throughout a 24 h incubation period, cGMP-elevating agents were present throughout this time. In the studies presented here, the cGMP-elevating agents were removed at the end of the 24 h pre-treatment period, and cGMP levels were below the limit of detection at this time. Therefore, whether PKG levels would remain sufficiently elevated and active at the mitochondrial pore in the absence of continued cGMP production is not clear.

Alternatively, cGMP/PKG may trigger downstream signalling events to provide longer-term protection against apoptosis. For example, non-toxic doses of NO have been demonstrated to protect murine macrophages against subsequent apoptotic concentraions of NO via activation of NF-κB and ensuing COX-2 expression (von Knethen and Brune 1997; von Knethen et al. 1999). Although the studies quoted above did not investigate the absolute requirement for cGMP in this protection, it is possible that cGMP activates PKG, which in turn triggers a signalling cascade involving mitogen-activated protein (MAP) kinases as the link between cGMP, and ultimately, NF-κB activation and COX-2 expression. Two such signalling cascades have recently been demonstrated in human pulmonary epithelial cells. The first involves cGMP/PKG triggering complicated sequential signalling pathways involving activation of multiple enzymes and kinases, including GTPases, phosphoinositide-3-OH-kinase (PI3K), and Akt, which ultimately act to phosphorylate and therefore, remove, the inhibitory subunit, IκBα, from NF-κB. Removal of this inhibitory subunit generates free NF-κB which is then able to translocate to the nucleus to cause COX-2 transcription (figure 6.5; Chang et al. 2004). Alternatively, the second pathway involves cGMP/PKG leading to COX-2 expression via activation of protein kinase C (PKC), followed by p44/42 MAPK activation (figure 6.5; Chang et al. 2002; Chang et al. 2004). However, since the actions of NO in relation to apoptosis are cell-type specific, this would need thorough investigation in human monocyte-derived macrophages before such a pathway could be identified as the protective mechanism. Indeed, Yoshioka et al reported that specific p38 MAPK inhibitors did not affect NO-mediated

cytoprotection in murine macrophages (Yoshioka et al. 2003), although the effect of specific p44/42 MAPK inhibitors were not investigated.

Clearly, the mechanism by which cGMP is able to prime cells against subsequent apoptotic agents in the relatively long-term is likely to involve complicated downstream signalling pathways, which require thorough investigation

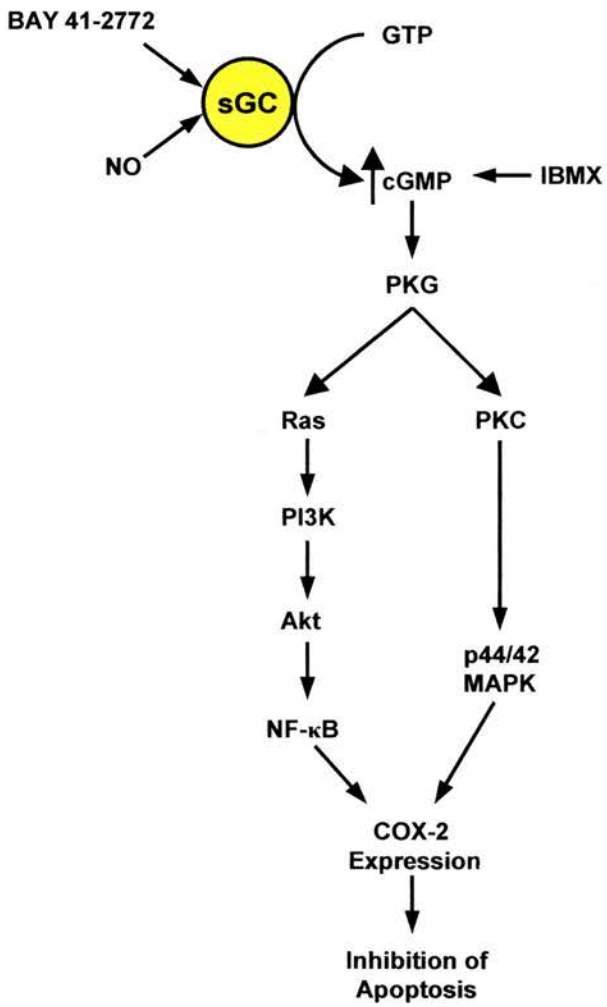

Figure 6.5 Possible Signalling Cascades in NO-Mediated Protection Against Apoptosis

Schematic representation of the possible signalling cascade triggered by cGMP/PKG. PKG activates various kinases which ultimately remove the inhibitory sub-unit of NF-κB, allowing it to translocate to nucleus and ultimately cause COX-2 expression. Adapted from (Chang et al. 2004)

In rodent macrophages, the presence of NO-mediated protective mechanism(s) may be a self-defence against the high NO concentrations produced by these cells themselves. This is unlikely to be the case in human monocyte-derived macrophages because the production of NO by human macrophages is either exceedingly low, or absent (chapter five). In studies demonstrating this phenomenon in murine macrophages (von Knethen et al. 1999; Yoshioka et al. 2003), both the non-toxic (~100 μM) and toxic doses of NO (3-4 mM) are likely to be far higher than normal NO producing capacity of the endothelium, although they may reflect supraphysiological iNOS-generated concentrations in murine cells under pathological conditions (von Knethen et al. 1999; Yoshioka et al. 2003). The results presented here demonstrate that not only do human macrophages possess such protective mechanisms, but also that they are active at far lower NO concentrations (~2.5 μM; chapter three). This lower dose of NO is predicted to be a more realistically physiological NO concentration that is closer to NO-producing capacity of the healthy endothelium. Although the presence of NO-mediated protective mechanisms is unlikely to be a self-defence mechanism in human macrophages, it may initially be an indirect, protective anti-inflammatory mechanism. During atherosclerosis, the net production of NO by the endothelium decreases (Chowienczyk et al. 1992; Cooper and Heagerty 1998). Although in the later stages of atherosclerosis this may contribute to the atherogenic process by promoting vasoconstriction and platelet aggregation, in the earliest stages of atherogenesis decreasing NO production may be beneficial. Allowing apoptosis to proceed by removing NO-mediated inhibition of apoptosis may be an attempt to resolve the vascular inflammation, now accepted to be a major component of atherosclerosis

(Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002). In keeping with this hypothesis is the observation of apoptotic macrophages in the plaques of both human excised carotid endarterectomy specimens and experimental models of atherosclerosis (Han et al. 1995; Bjorkerud and Bjorkerud 1996; Haunstetter and Izumo 1998), although whether this had been brought about by decreased NO bioavailability is not known.

If decreasing NO output from the endothelium is an anti-inflammatory defence, delivering NO later in atherosclerosis might be expected to exacerbate vascular inflammation. However, increasing NO bioavailability by dietary supplementation of L-Arg increased the number of apoptotic macrophages present in vascular lesions and this was associated with plaque regression in a rabbit model of atherosclerosis (Wang et al. 1999). Induction of apoptosis in this setting occurred independently of cGMP-signalling, suggesting that a separate mechanism is responsible for triggering apoptosis under these conditions. Given that the environment of the atherosclerotic plaque is likely to be under considerable oxidative stress, the additional NO generated by supplementary L-Arg would combine rapidly with O_2^-, generating $ONOO^-$ and, therefore, $ONOO^-$ may be the apoptotic trigger, rather than NO *per se*. Thus, delivering NO in an appropriate chemical form to induce cGMP-independent apoptosis later in atherosclerosis could be exploited to induce apoptosis and reduce atheroma, without initiating NO-mediated inhibition of apoptosis. Although it is not yet clear whether $ONOO^-$ -induced apoptosis involves cGMP signalling, the absence of detectable cGMP in cells treated for 24 h with GEA-3162 suggests that cGMP-independent pathways are responsible for $ONOO^-$ -induced apoptosis.

NO and elevated cGMP levels were unable to protect BAoSMC against $ONOO^-$ -induced necrotic cell death. This is perhaps unsurprising because, unlike apoptosis, necrosis is not a regulated process, so there are less potential regulatory targets for the control of necrosis.

The NO-independent sGC activator, YC-1, has previously been shown to inhibit NO-induced apoptosis in rat vascular smooth muscle cells (VSMC; Pan et al. 2004)). However, although the study quoted above did not measure NO, the concentration of NO donor used to induce apoptosis (SNP; 1mM) is likely to have generated significantly higher NOx concentrations than those used in the current study. The data presented here, and in previous chapters, demonstrates that lower concentrations of NO do not induce apoptosis in BAoSMC, and that $ONOO^-$ induces necrosis in this cell type. The results presented here do not necessarily demonstrate that cGMP is incapable of protecting VSMCs against apoptosis, but do show that if such protective mechanism exist in VSMCs they are not active at physiologically relevant NO concentrations, and do not protect cells against necrotic cell death.

Preserving VSMCs is of fundamental importance to maintaining the integrity of the atherosclerotic plaque cap, hence preventing plaque rupture and the acute clinical consequences of atherosclerosis, such as myocardial infarction and stroke. Although the determinants of plaque rupture have yet to be been fully identified, such events are associated with a reduction in the thickness and VSMC content of the plaque cap (Bauriedel et al. 1999; Leskinen et al. 2003). Therefore, it would seem that the best therapeutic approach for the treatment of atherosclerosis would see apoptosis and phagocytic clearance of lipid-laden inflammatory cells resident in the plaque core, whilst maintaining the cap by protecting the VSMCs from cell death

(either apoptosis or necrosis). The data presented here demonstrate that delivering NO or NO-related species late in atherosclerosis may not be the best approach to this theoretical strategy. Although the NO-related species, $ONOO^-$ may have beneficial effects by inducing apoptosis in inflammatory cells, the cost of triggering necrosis in the VSMC population of the cap is likely to be too great in terms of plaque stability, and this cannot be off set by a two pronged approach aimed at elevating cGMP in VSMC prior to delivery of $ONOO^-$ to induce inflammatory cell apoptosis.

These data also go some way to explain the disparate actions of NO and NO-related species in inducing apoptosis. It appears from these studies that, under normal physiological conditions, NO acts to inhibit apoptosis via an elevation in cGMP levels, which subsequently triggers downstream signalling cascades and, possibly, the transcription of anti-apoptotic genes. Alternatively, supraphysiological concentrations of NO generated under pathological conditions induce apoptosis, but only following prior conversion to the NO-related species, $ONOO^-$.

Chapter Seven

General Discussion & Future Directions

7. General Discussion and Future Directions

7.1 Introduction

Despite decades of research, the clinical consequences of atherosclerosis remain a major cause of morbidity and mortality in industrialised nations (Callow 2002; The American Heart Association Statistical Update 2005), and the mechanisms underlying this widespread condition are still not fully understood. Atherosclerosis is characterised by the formation of lipid rich plaques in the sub-endothelial space of large conduit blood vessels (Ross 1993; Davies 1997), and it is now widely accepted that there is a chronic inflammatory component to the condition (Ross 1999a; Ross 1999b; Libby 2002; Libby et al. 2002). Atherosclerosis develops over a period of several decades and although atherosclerosis may be extensive by middle age, the majority of such plaques are a latent presence in the vessel wall and remain asymptomatic. However, a minority of plaques rupture, giving rise to the acute cardiovascular syndromes such as unstable angina pectoris, myocardial infarction and stroke (Davies 1995; Dalager-Pedersen et al. 1998; Gutstein and Fuster 1999; Zhou et al. 1999; Corti and Badimon 2002; Mitra et al. 2004). What causes a given plaque to rupture is still not fully understood, but current evidence points towards inflammatory processes occurring within the plaque. Therefore, an urgent clinical need exists to understand the processes underlying plaque rupture and to identify

inflamed plaques so that drug intervention can be selectively targeted towards those plaques with the greatest propensity to rupture.

Given that apoptosis of inflammatory cells is thought to be critical to the successful resolution of the inflammatory response (Haslett 1997; Maderna and Godson 2003), and that inflammatory processes are implicated in both the development of atherosclerotic plaques and their rupture, manipulation of inflammatory cell apoptosis may hold the key to stabilising plaques, limiting their development and preventing their rupture. However, because of the complicated nature of atherosclerosis and the many different cell types present within plaques, it is crucial to ensure that apoptosis is manipulated in a highly controlled manner. The challenge, therefore, is to be able to distinguish, and selectively target, cells types responsible for the demise of the plaque and whilst preserving those cell types involved in maintaining plaque stability.

Use of NO as a means of inducing apoptosis in order to limit plaque development and stabilise pre-existing lesions is a particularly attractive prospect because in addition to aiding resolution of vascular inflammation via apoptosis, NO possesses various additional anti-atherogenic properties such as inhibiting platelet activation and aggregation, and promoting vasodilatation (Moncada et al. 1991; Ignarro et al. 1999). However, NO radical readily forms other NO-related species that have biological properties of their own, and these can be quite different to NO *per se*. Formation of such NO-related species may account for the paradoxical actions of NO reported in various settings. It is, therefore, crucial to understand the precise nature of the NO-related species responsible for a particular process, and in

order to use NO therapeutically, it is essential to ensure that NO is delivered in the appropriate chemical form to induce the desired response.

This thesis has used a variety of synthetic putative NO donor compounds to investigate differences between the actions of NO and NO-related species in cell proliferation and viability in two of the major cell types of atherosclerotic plaques.

7.2 Summary

In order to investigate the disparate actions of NO and NO-related species, exhaustive efforts were made to fully characterise the NO and NO-related species generated by the putative NO donor compounds used in subsequent biological investigations (chapter three). Using a combination of an isolated NO electrode and EPR spectroscopy, diazeniumdiolate compounds were identified as fast and slow release NO radical donors, DEA/NO and DETA/NO respectively. Contrary to initial studies published on GEA-3162 (Kankaanranta et al. 1996; Holm et al. 1998), this compound was found to co-generate NO and O_2^-, so should be regarded as a $ONOO^-$ generator. Although the methods in chapter three did not identify the specific NO-related species liberated by RS-N=O compounds, they did rule out NO radical and oxidising species as the main decomposition product of this class of compound. This observation, considered in combination with previously published data on RS-N=O, imply that transnitrosation reactions (transfer of NO^+) are likely to account for the biological actions of this group of compounds.

Somewhat surprisingly, the fundamental chemical composition of the solution into which NO donor compounds were introduced significantly affected the

concentration of NO generated, with a component of DMEM accelerating the decomposition of all classes of compound. Although the specific component of DMEM responsible for this acceleration was not identified, this observation underlines the absolute requirement to conduct this type of preliminary characterisation study in conditions closely modelling those used in subsequent biological experiments. Furthermore, they suggest that the fate of NO *in vivo* might be hard to predict, and therefore, it is crucial to be absolutely certain that any NO donor compounds used therapeutically are able to deliver the precise NO-related species necessary to achieve the desired response.

Studies examining the role of NO and NO-related species at concentrations that might reasonably be expected *in vivo*, surprisingly demonstrated that only the NO-related species, $ONOO^-$, had any impact on cell viability (chapters 4 & 5). $ONOO^-$ was able to induce apoptosis in monocytes and monocyte-derived macrophages, but also induced significant necrosis in VSMC. Although, $ONOO^-$ appeared to inhibit VSMC proliferation, on further investigation this was found to be due to a decrease in cell number as a result of necrotic cell death, and only a prolonged release of NO radical was able to genuinely inhibit VSMC proliferation. Contrary to previous reports (Assender et al. 1992; Newby et al. 1992; Garg and Hassid 1993; Yu et al. 1997; Jeremy et al. 1999), RS-N=O and short exposure to NO radical and were unable to affect proliferation in VSMCs. Similarly, NO radical and RS-N=O did not induce apoptosis in VSMCs, monocytes or monocyte-derived macrophages (figure 7.1 shows a summary of the findings of these experiments). The main reasons for this contradiction between previously published data and that presented in this thesis are likely to relate to both the concentrations of NO donor

compounds involved and species origin of the cells examined. Most studies reporting anti-mitogenic and pro-apoptotic actions of NO use significantly greater concentrations of NO donor compound (usually >1mM) than those used in this thesis (Albina et al. 1993; Sarih et al. 1993; Muhl et al. 1996; Bennett and Boyle 1998). Such high concentrations of donor compound are likely to generate concentrations of NO far in excess of the NO producing capacity of the healthy endothelium. Previously published studies demonstrating that NO is capable of inducing macrophage apoptosis have been conducted in rodent cells (both cell lines and isolated peritoneal cells) rather than freshly isolated, terminally differentiated, human macrophages used in thesis. Therefore, it might be argued that the results presented in this thesis more realistically represent the *in vivo* actions of NO in humans, and that many of the properties previously ascribed to NO do not occur under normal physiological conditions.

Finally, experiments in chapter six demonstrated what might be considered a physiologically relevant concentration of NO is able to trigger cGMP-dependent protection against $ONOO^-$ -induced apoptosis in human monocyte- derived macrophages, but is unable to protect VSMCs against $ONOO^-$ -induced necrosis (figure 7.1). This phenomenon has been previously reported in rodent macrophages where it has been proposed as a self-defence mechanism against the high iNOS-derived NO generated by rodent cells (Albina et al. 1993; Sarih et al. 1993; Muhl et al. 1996; Bennett and Boyle 1998; von Knethen et al. 1999; Yoshioka et al. 2003; Pan et al. 2004). However, the cGMP-dependent protection against apoptosis induced in human cells is unlikely to serve this purpose, because as experiments in chapter five demonstrated, the monocyte-derived macrophages used in experiments

in this thesis do not generate high levels of NO. In fact, one might predict that a self-defence mechanism against high NO concentrations would be more likely, and more necessary, in VSMCs, given that this cell type both produce NO themselves, and are constantly exposed to NO generated by the endothelium. However, as discussed in chapter four, VSMCs might be susceptible to apoptotic stimuli when the have undergone phenotype alteration to the proliferative phenotype thought to dominate in the VSMC population of atherosclerotic plaques. Therefore, it might be that in healthy vessels VSMCs are exposed to only low concentrations of NO and are less sensitive to apoptotic stimuli so don't require such a self-defence mechanism.

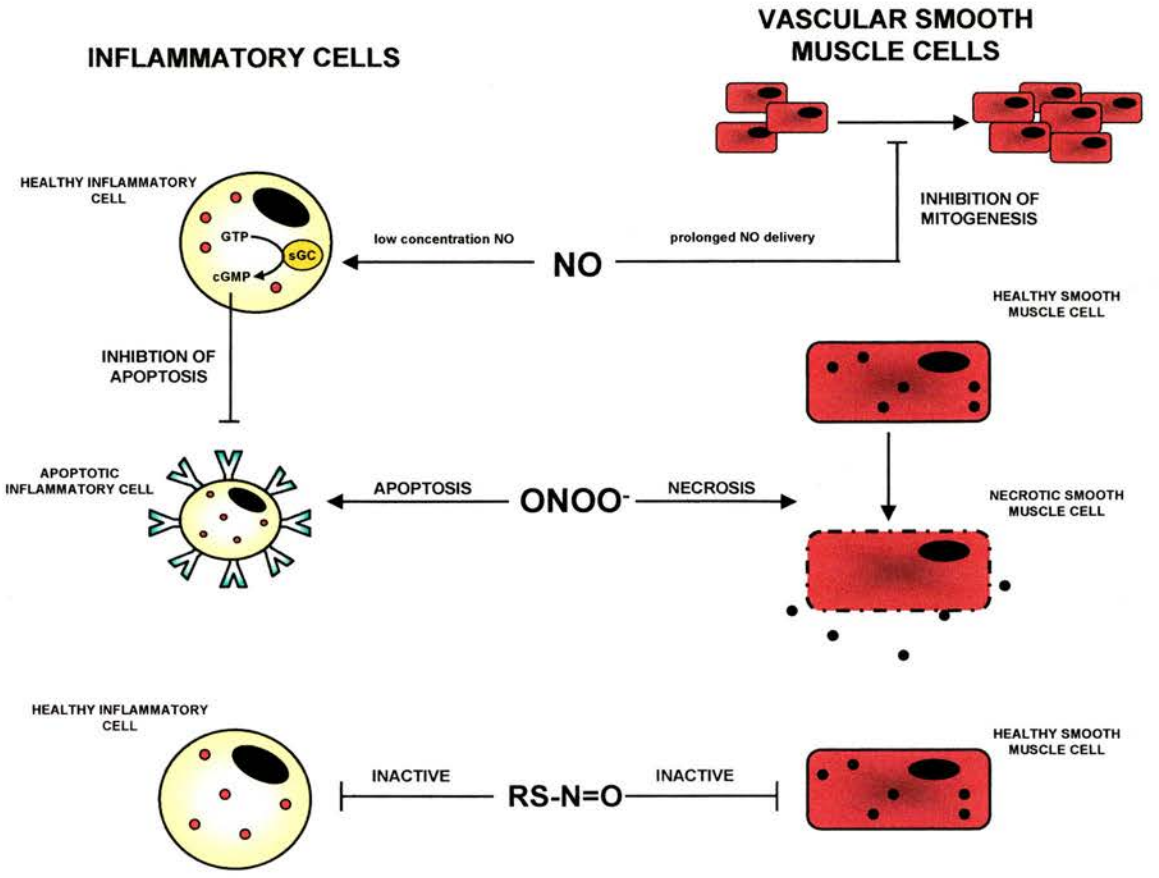

Figure 7.1 Schematic Representation of the Actions of NO and NO-Related Species in Human Monocyte-Derived Macrophages and BAoSMC

$ONOO^-$ induces apoptosis in monocyte-derived macrophages and necrosis in BAoSMC. A prolonged delivery of NO radical inhibitions cell proliferation in BAoSMC. Low concentrations of NO are able to active cGMP-dependent protection against $ONOO^-$ -induced apoptosis in monocyte-derived macrophages, but are unable to protect BAoSMC against $ONOO^-$ -induced necrosis. NO radical does not induce apoptosis in either cell type, and RS-N=O compounds have no impact on cell proliferation or apoptosis in monocyte-derived macrophages or BAoSMC.

7.3 Clinical Implications for the Treatment of Atherosclerosis

Although $ONOO^-$ -mediated induction of apoptosis in inflammatory cells within the plaque may be desirable as a means to reduce the inflammatory load in a plaque and prevent rupture during the later stages of atherosclerosis, the cost of decreasing the VSMC population of the plaque cap $ONOO^-$ -mediated necrosis is likely to be too great in terms of de-stabilising the plaque and promoting rupture. Unfortunately, these findings suggest that use of NO and NO-related species may not be an appropriate therapy for atherosclerosis. However, the future development of compounds with a greater degree of cell specificity may enable macrophage-specific $ONOO^-$ generators to be used to this end. The macrophages resident in the plaque core retain oxidised lipids and develop into lipid-laden foam cells. Therefore, one possible means of achieving this higher degree of selectivity may be to exploit the lipid nature of the foam cells by developing lipophilic compounds that would accumulate in the core where selective induction of apoptosis might be beneficial. Given that NO radical is non-toxic towards VSMCs it might be possible to use lipophilic NO donor drugs to deliver NO directly to the plaque core where the environment of elevated oxidative stress would likely result in the formation of $ONOO^-$. This might enable selective induction of apoptosis in the inflammatory cells of the core whilst leaving the population of VSMC population unaffected. However, two notes of caution must be borne in mind when considering this possibility. Firstly, the accumulation of lipids within macrophages changes the nature of the cell

and it is unclear whether foam cells would respond in the same way as monocyte-derived macrophages. Secondly, the success of inducing apoptosis to resolve inflammation critically depends on efficient phagocytosis of apoptotic cells. The identity of the cells responsible for the phagocytic clearance of apoptotic macrophages remains to be established, but given that $ONOO^-$ induces apoptosis both in undifferentiated monocytes and other types of inflammatory cells, such as neutrophils, it is likely that $ONOO^-$ will also induce apoptosis in the population of phagocytes. Removing phagocytosis will exacerbate the inflammatory response as a result of secondary necrosis.

Unfortunately, the ability of NO to induce cGMP-dependent inhibition of $ONOO^-$ -mediated apoptosis in monocyte-derived macrophages, but not VSMCs means that a two-pronged strategy of delivering low concentrations of NO in combination with $ONOO^-$ would be unsuccessful in terms of plaque stability.

7.4 Future Directions

This thesis has revealed some interesting insights into the disparate actions of NO and NO-related species that might help explain some of the paradoxical actions of NO previously reported. Whilst the effects of NO and NO-related species have been explored in this thesis, the constraints of time did not permit investigations into the mechanisms of these process.

7.4.1 $ONOO^-$ -Induced Apoptosis

Because apoptosis is such a tightly regulated, and cell specific process, it is difficult to make comparisons about the mechanism of apoptosis between different cell types. Therefore, the mechanism of $ONOO^-$ -induced apoptosis should be fully investigated in human monocyte-macrophages. A crucial first step in this process should be to identify the precise mediator of apoptosis. Results in chapter five demonstrated that SOD increased $ONOO^-$ -induced apoptosis, suggesting that H_2O_2 is the final effector of apoptosis. This could be determined relatively simply by treating cells with GEA-3162 in the presence of a combination of SOD and catalase followed by flow cyotmetric analysis.

It would be of interest to identify the downstream mechanism of $ONOO^-$ -induced apoptosis. A reasonable starting point would be to investigate if mitochondrial depolarisation and cytochrome-*c* release precede apoptosis in human monocyte-derived macrophages, as they have been reported to do in NO-induced apoptosis in murine macrophages (Brown and Borutaite 1999; Hortelano et al. 1999; Bal-Price and Brown 2000; Brown and Borutaite 2001; Brown and Borutaite 2002; Maneiro et al. 2005). This can be done by use of fluorescent mitochondrial permeable dyes such as JC-1, which can be detected by flow cytometry or visualised under the fluorescent microscope. There are some technical considerations in conducting this experiment in human monocyte-derived macrophages because these cells adhere to tissue culture plates and are usually removed from the plates prior to flow cytometric analysis by trypsin:EDTA solution. This is likely to disrupt membrane potentials due to chelation of metal ions and so may give false results.

One possible way to overcome this difficulty would be to culture the cells in Teflon™ pots, which have been found by our group to resist cellular adhesion.

Examining the ratio of pro- to anti-apoptotic Bcl-2 proteins, and levels of various caspase isoenzymes could further elucidate the mechanism of apoptosis. This could be done by Western blotting techniques and, depending on the availability of appropriate antibodies, the localisation of Bcl-2 proteins and caspases could be visualised by immunofluorescence.

In the context of atherosclerosis, it would be interesting to investigate if the pro-apoptotic actions of $ONOO^-$ apply to foam cells, as well as to macrophages. Given that `foam cells are the major cellular component of the plaque core, inducing apoptosis and subsequent phagocytosis in this cell type might be advantageous both in terms of reducing the size, and the thrombogenicity, of the plaque. Lipid loading is likely to change the characteristics of the cell considerably and therefore, they might have altered responses to apoptotic stimuli.

7.4.2 NO Production Human Macrophages

The ability of human macrophages to generate NO has been debated for some time (Albina 1995; Schneemann and Schoedon 2002). The results from thesis (chapter five) demonstrate that NO production from LPS-stimulated monocyte-derived macrophages isolated from peripheral blood is exceedingly low or absent. However, as discussed in chapter five the stimulant used may have been insufficient. It is worth pursuing this issue further for a definitive answer as whether or not these cells produce NO, because this could have implications for various inflammatory diseases, including atherosclerosis. Treating the cells with LPS in combination with

a 'cocktail' of cytokines including TNF-α and interferon gamma (IFN-γ) may provide adequate stimulation. Additionally, the cells should be analysed for NOS expression by Western Blot.

7.4.3 cGMP-Dependent Protection Against Apoptosis

The mechanism by which cGMP is able to confer protection on human monocyte-derived macrophages should be further investigated in order to pin point the precise signalling pathways involved. A number of potential pathways including p38 MAP kinases, NF-κB signalling and protein kinase C activation leading to COX-2 expression were discussed in chapter six. Commercially available kinase assays could be used to assess the activity of various potential kinases in these pathways. Western blotting could be used to detect kinase enzymes in their unphosphorylated (inactive) and phosphorylated forms (active) forms. These pathways could also be investigated pharmacologically using specific inhibitors of various potential signalling enzymes. Finally, COX-2 as the ultimate inhibitor of apoptosis could be confirmed by use of selective COX-2 inhibitors to abrogate cGMP-dependent protection against apoptosis.

7.4.4 Phagocytic Clearance of Apoptotic Macrophages

Use of apoptosis to aid resolution of vascular inflammation in atherosclerosis critically depends on the successful clearance of apoptotic cells from the site of inflammation. The identity of the cells responsible for clearing apoptotic macrophages has not been identified. It would be interesting to investigate if macrophages themselves phagocytose populations of apoptotic macrophages, or

whether this is done by a sub-set of specialised phagocytes. This could be investigated by inducing apoptosis in a set of monocyte-derived macrophages labelled with a fluorescent marker, and then introducing a second set of healthy macrophages, labelled with a different fluorescent marker, to the culture. The cells could then be analysed by flow cytometry and any healthy cells that had ingested apoptotic cells would be double labelled.

Overall, these studies should provide further insights into the mechanisms of action of NO and NO-related species. Identifying downstream signalling pathways may enable specific pathways, or parts of pathways, to be targeted therapeutically. Results presented in this thesis somewhat disappointingly suggest that NO or NO-related species may not be able to selectively induce apoptosis in a particular cell type during atherosclerosis without causing serious detrimental effects in other cell types. However, identification of the downstream signalling pathways and ultimate effectors of apoptosis may enable apoptotic pathways to be triggered whilst by-passing the detrimental effects in other cell types. Similarly, the development of cell-specific NO donor compounds may allow selective targeting of pro-apoptotic NO-related species directly to the macrophages within the plaque core, whilst preserving the integrity of the VSMC cap.

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Appendix One
Media Formulation

1. DMEM

COMPONENTS	Molecular Weight	Concentration (mg/L)	Molarity (mM)
Amino Acids			
Glycine	75	30	0.400
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Histidine hydrochloride-H2O	210	42	0.200
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.400
L-Serine	105	42	0.400
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine	181	72	0.398
L-Valine	117	94	0.803
Vitamins			
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic Acid	441	4	0.00907
i-Inositol	180	7.2	0.0400
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	4	0.0119
Inorganic Salts			
Calcium Chloride ($CaCl_2$-$2H_2O$)	147	264	1.80
Ferric Nitrate ($Fe(NO_3)3"9H_2O$)	404	0.1	0.000248
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	246	200	0.813
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate ($NaHCO_3$)	84	3700	44.05
Sodium Chloride (NaCl)	58	6400	110.34
Sodium Phosphate monobasic (NaH_2PO_4-$2H_2O$)	154	141	0.916
Other Components			
D-Glucose (Dextrose)	180	4500	25.00
Phenol Red	376.4	15	0.0399
Sodium Pyruvate			

2. IMDM Formulation

COMPONENTS	Molecular Weight	Concentration (mg/L)	Molarity (mM)
Amino Acids			
Glycine	75	30	0.400
L-Alanine	89	25	0.281
L-Arginine hydrochloride	211	84	0.398
L-Asparagine-H2O	150	28.4	0.189
L-Aspartic acid	133	30	0.226
L-Cystine	240	70	0.292
L-Glutamic Acid	147	75	0.510
L-Glutamine	146	584	4.00
L-Histidine hydrochloride-H2O	210	42	0.200
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.400
L-Proline	115	40	0.348
L-Serine	105	42	0.400
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine disodium salt	225	104	0.462
L-Valine	117	94	0.803
Vitamins			
Biotin	244	0.013	0.0000533
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic Acid	441	4	0.00907
i-Inositol	180	7.2	0.0400
Niacinamide	122	4	0.0328
Pyridoxal hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	4	0.0119
Vitamin B12	1355	0.013	0.0000096

IMDM Formulation

Cont.

Components	Molecular Weight	Concentration (mg/L)	Molarity (mM)
Inorganic Salts			
Calcium Chloride ($CaCl_2$-$2H_2O$)	147	219	1.49
Magnesium Sulfate ($MgSO_4$-$2H_2O$	176	200	1.14
Potassium Chloride (KCl)	75	330	4.40
Potassium Nitrate (KNO_3)	101	0.076	0.000752
Sodium Bicarbonate ($NaHCO_3$)	84	3024	36.00
Sodium Chloride (NaCl)	58	4505	77.67
Sodium Phosphate monobasic (NaH_2PO_4-$2H_2O$)	156	141	0.904
Sodium Selenite (Na_2SeO_3-$5H_20$)	263	0.0173	0.0000658
Other Components			
D-Glucose (Dextrose)	180	4500	25.00
HEPES	238	5958	25.03
Phenol Red	376.4	15	0.0399
Sodium Pyruvate	110	110	1.000

Appendix Two

Publications

Nitric oxide and the resolution of inflammation: implications for atherosclerosis

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The ubiquitous free radical, nitric oxide (NO), plays an important role in many biological processes including the regulation of the inflammatory response. Alterations in NO synthesis by endogenous systems likely influence inflammatory processes occurring in a wide range of diseases including many in the cardiovascular system (e.g. atherosclerosis). Progression of inflammatory conditions depends not only upon the recruitment and activation of inflammatory cells but also upon their subsequent removal from the inflammatory milieu. Apoptosis, or programmed cell death, is a fundamental process regulating inflammatory cell survival and is critically involved in ensuring the successful resolution of an inflammatory response. Apoptosis results in shutdown of secretory pathways and renders effete, but potentially highly histotoxic, cells instantly recognisable for non-inflammatory clearance by phagocytes (e.g., macrophages). However, dysregulation of apoptosis and phagocytic clearance mechanisms can have drastic consequences for development and resolution of inflammatory processes. In this review we highlight the complexities of NO-mediated regulation of inflammatory cell apoptosis and clearance by phagocytes and discuss the molecular mechanisms controlling these NO mediated effects. We believe that manipulation of pathways involving NO may have previously unrecognised therapeutic potential for limiting or resolving inflammatory and cardiovascular disease.

Key words: nitric oxide - apoptosis - inflammation - resolution

The inorganic free radical, nitric oxide (NO), was first identified as an endothelium-derived endogenous messenger responsible for the regulation of vascular tone (Furchgott & Zawadzki 1980, Palmer et al. 1987). However, since then it has become clear that NO is the signalling molecule responsible for several diverse physiological and pathophysiological processes. Synthesised from L-arginine by three isoforms of the enzyme nitric oxide synthase (NOS), NO is now known to control vascular smooth muscle tone, inhibit platelet and inflammatory cell adhesion and activation, and to be a transmitter at non-adrenergic non-cholinergic (NANC) synapses (Moncada et al. 1991, Quinn et al. 1995). Recent studies have revealed that NO can also modulate apoptosis, or programmed cell death, in a variety of cell types, including human inflammatory cells (Taylor et al. 2003). Apoptosis of inflammatory cells is a highly regulated process whereby cellular death occurs without the disruption of the cell membrane and subsequent release of the pro-inflammatory and histotoxic contents of the dying cell (Haslett 1997, Rossi et al. 2003). Apoptotic cells are instantly recognised and ingested by phagocytes, such as macrophages, using mechanisms that down-regulate pro-inflammatory mediator release and increase the release of agents with anti-inflammatory potential from the ingesting cell (Meagher et al. 1992, Fadok et al. 1998, Liu et al. 1999). Hence, apoptosis represents a non-inflammatory mechanism to remove potentially damaging pro-inflammatory cells from the site of inflammation and is therefore critical to the successful resolution of the inflammatory response. Pharmacological manipulation of the rate of apoptosis in inflammatory cells, such as granulocytes and macrophages, may represent a potential therapeutic strategy for the treatment of chronic inflammatory disorders (Ward et al. 1999, Gilroy et al. 2004).

NO can be both pro- and anti-apoptotic, depending on local concentrations and the specific cell type in question (Quinn et al. 1995, Kim et al. 1999, Taylor et al. 2003). Current evidence suggests that lower concentrations of NO produced by the constitutive endothelial and neuronal isoforms of NOS (eNOS and nNOS) are cytoprotective, whilst supraphysiological concentrations produced by the inducible NOS isoform (iNOS) trigger cell death (Nicotera et al. 1997). This paradox may be explained, at least in part, by the free radical nature of NO and hence the ease with which it will react with other radicals, particularly reactive oxygen species, present in the milieu to form various NO-related species in vivo. For example, NO combines rapidly with inflammatory cell derived superoxide anions (O_2^-) to form highly cytotoxic peroxynitrite ($ONOO^-$) (Maxwell & Lip 1997).

NO as a mediator of inflammatory cell apoptosis

The pro- and anti-apoptotic actions of NO have been well documented in many cell systems. For example, high concentrations of either exogenous or endogenous iNOS-derived NO have been shown to induce apoptosis in murine macrophage cell lines (Albina et al. 1993, Sarih et al. 1993). However, pre-treatment with low concentrations of exogenous NO protects RAW 264 cells against cell death upon subsequent exposure to higher concentra-

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tions of NO which would normally be cytotoxic (Yoshioka et al. 2003). However, despite the apparent reduced capacity of human macrophages in comparison to murine macrophages (Albina 1995, Schneemann & Schoedon 2002), to generate iNOS derived NO (Thomassen & Kavuru 2001), human macrophages do undergo apoptosis in response to exogenous NO. For example, the NO donors, S-nitrosoglutathione (GSNO), and spermine diazenium diolate (SPER/NO) induce apoptosis in primary human monocyte-derived macrophages (von Knethen et al. 1999). Exogenously delivered NO from NO donors (e.g., sodium nitroprusside; SNP and GSNO) induce apoptosis in human neutrophils (Fortenberry et al. 1999, Singhal et al. 1999). However, it has also been established that NO may have anti-apoptotic potential in neutrophils; low concentrations of NO generated from the spontaneous NO donors, SPER/NO and DEA/NO, reduce the rate of neutrophil apoptosis (Taylor et al. 2001). In contrast, the same study showed that the oxatriazole derivative, GEA-3162, at equivalent concentrations produced no such inhibition. However, it was demonstrated that GEA-3162 decomposes to co-generate both NO and O_2^-, which then react to form $ONOO^-$ (Taylor et al. 2004). This suggests that the pro- or anti-apoptotic effects of NO may be critically governed by the specific NO-related species generated.

Interestingly, the production of $ONOO^-$ may be of particular importance at sites of inflammation where the concentration of reactive oxygen species is likely to be elevated (Crow & Beckman 1995). However, the precise role of $ONOO^-$ in inflammatory cell apoptosis remains to be fully elucidated. There is some evidence to suggest that $ONOO^-$ at high concentrations increases apoptosis in murine RAW 264.7 cells (Sandoval et al. 1997), whilst at lower concentrations it may have a protective effect against lipopolysaccharide (LPS) and interferon (IFN)-γ-induced apoptosis in these cells (Scivittaro et al. 1997). A scavenger of $ONOO^-$, uric acid, had no effect on apoptosis induced by the NO donors GSNO or SPER/NO in RAW 264.7 macrophages, but abolished apoptosis induced by the $ONOO^-$ generator SIN-1, suggesting that $ONOO^-$ is a mediator of apoptosis, at least not in this cell type (Brockhaus & Brune 1999).

As is the case with macrophages, there are conflicting reports about the ability of $ONOO^-$ to induce or suppress apoptosis in neutrophils. Several investigators have demonstrated that SIN-1 and GEA-3162 increases the rate of apoptosis in human neutrophils (Blaylock et al. 1998, Ward et al. 2000, Taylor et al. 2004). Conversely, Blaylock et al. (1998) reported SIN-1 produced no significant increase in neutrophil apoptosis. However, this may be due to experimental differences and the exact amounts of $ONOO^-$ present rather than a true difference in the effect of $ONOO^-$ (Taylor et al. 2003).

NO and apoptosis in the resolution of inflammation

The ability of NO to induce apoptosis is particularly relevant during the resolution phase of inflammation. In a mouse model of kidney inflammation, activated macrophages have been shown to induce apoptosis in neighbouring mesangial cells prior to their ingestion by phagocytes (Duffield et al. 2000). The ability of these activated macrophages to induce apoptosis is greatly reduced in the presence of the NOS inhibitor N-É-monomethyl-L-arginine (L-NMMA), suggesting that macrophage-directed apoptosis of mesangial cell apoptosis occurs via a NO-dependent mechanism (Duffield et al. 2001). Similarly, several studies have demonstrated that activated macrophages infiltrating murine tumours induce apoptosis via a NO-dependent pathway in both activated anti-tumour T cells and in the tumour cells themselves (Saio et al. 2001, Chattopadhyay et al. 2002). Thus, it appears that macrophages have the capacity to induce apoptosis of nearby cells by the liberation of NO to enhance the clearance of apoptotic cells and thereby promote the resolution phase of inflammation (Figure).

Mechanism of action of NO

The classical pathway by which NO exerts many of its actions is via activation of the enzyme soluble guanylate cyclase (sGC) (Moncada et al. 1991) and resultant conversion of guanosine 5'-triphosphate (GTP) to the second messenger 3', 5'-cyclic guanosine monophosphate (cGMP) (Ignarro et al. 1999). However, recent studies have established that NO can also act via cGMP-independent pathways in various systems, particularly during the inhibition of platelet aggregation and regulation of inflammatory cell apoptosis (Gordge et al. 1998, Sogo et al. 2000, Ward et al. 2000, Crane et al. 2002).

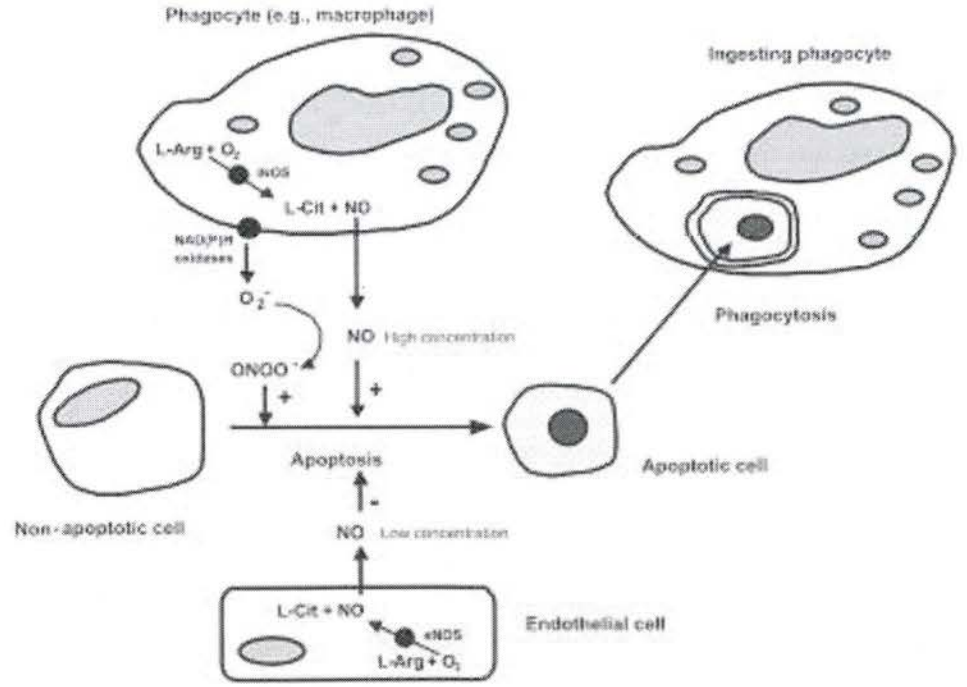
It is generally thought that lower concentrations of NO inhibit apoptosis via cGMP-dependent mechanisms, whilst higher concentrations are cytotoxic on account of cGMP-independent signalling. For example, Yoshoka et al. (2003) demonstrated that pre-treatment of RAW 264 cells with a low concentration of the NO donor SNP, inhibited cell death upon subsequent exposure to higher concentrations of NO. This protection was negated in the presence of sGC inhibitors and could be mimicked by cGMP analogues, suggesting that the cellular protection was conferred by cGMP.

Conversely, at higher concentrations, NO has been shown to induce apoptosis in rabbit macrophages – an effect which was unaffected by antagonism of cGMP-dependent kinases and not mimicked by cGMP analogues, suggesting that the pro-apoptotic action of NO is cGMP-independent (Wang et al. 1999). The peroxynitrite generators, SIN-1 and GEA-3162, have also been shown to produce a marked concentration-dependent induction of apoptosis in isolated human neutrophils (Ward et al. 2000). Again, this induction was unaffected by inhibitors of sGC, and cGMP analogues failed to elicit a pro-apoptotic response suggesting that a mechanism independent of cGMP signalling also featured in neutrophils. Interestingly, superoxide dismutase (SOD), the enzyme responsible for converting O_2^- to hydrogen peroxide (H_2O_2), antagonised the actions of SIN-1 and GEA-3162, whilst "authentic" peroxynitrite mimicked their effects. This result may, therefore, highlight the critical importance of NO-related species in determining an anti- or pro-apoptotic response, with the final outcome depending on the balance between reactive oxygen and nitrogen species.

Atherosclerosis

Atherosclerosis is a multi-factorial condition with a complicated aetiology, and, in combination with the associated cardiovascular syndromes, such as myocardial infarction and stroke, is a major cause of morbidity and mortality. However, it is now widely recognised that there is an inflammatory component to the disease pathogenesis and progression (Ross 1999a, b, Ludewig et al. 2002).

Atherosclerosis is characterised by the development of lipid-rich atherosclerotic plaques in the subendothelial space of conduit vessels, such as the coronary artery and aorta (Badimon et al. 1993). These plaques are usually eccentric, with the lipid rich core encapsulated by a fibrous, collagen-rich cap of smooth muscle cells and extracellular matrix (Davies 1997). The underlying causes of atherogenesis remain largely unknown, although a critical early stage is thought to be an insult to the endothelium, either physical or through oxidative stress. The consequences of this insult are multiple; firstly, in contrast to the situation in healthy endothelium, the injured endothelium becomes dysfunctional and production of NO by eNOS decreases, promoting vasoconstriction and platelet and inflammatory cell adhesion. Secondly, a protective inflammatory response is triggered. However, depending on the nature and duration of the insult, this protective response becomes excessive and over a period of years, comes to constitute the disease process itself (Ross 1999a, b). The inflammatory process begins with the expression of chemotactic and adhesion molecules for monocytes and lymphocytes, such as vascular cell adhesion molecule 1 (VCAM-1), on dysfunctional endothelial cells. Circulating monocytes adhere to the site of endothelial damage and translocate to the sub-endothelial space (Vogel 1997). Colony stimulating factors secreted from areas of endothelial damage induce monocytes to differentiate into macrophages, which then express scavenger receptors on their membranes, facilitating the internalisation of oxidised low density lipoprotein (ox-LDL). The accumulation of ox-LDL continues unchecked as, unlike LDL receptors, scavenger receptors are not down-regulated by cells in the cholesterol-replete state (Maxwell & Lip 1997). In this lipid-laden state, macrophages are known as foam cells and it is an aggregation of these foam cells in the vessel intima which form the earliest recognisable lesion of atherosclerosis – the fatty streak (Ross 1993). The plaque continues to grow via the accumulation of further macrophage foam cells and eventually becomes overlaid with a layer of smooth muscle cells forming a fibrous, collagen-rich cap. The cap serves to keep the highly thrombogenic contents of the plaque separate from the circulation. However, if the plaque cap is compromised and the contents exposed to the circulation, platelets are rapidly recruited and activated resulting in thrombus formation, leading to the more serious acute cardiovascular syndromes (Badimon et al. 1993).



High concentrations of nitric oxide (NO) synthesised by iNOS in phagocytes, such as macrophages, induce apoptosis in neighbouring cells. In addition, apoptosis can also be induced by $ONOO^-$ generated as O_2^- produced by phagocytes reacts with NO. Apoptotic cells are subsequently recognised and ingested by phagocytes, thus aiding the resolution of inflammation. Conversely, low concentrations of NO produced constitutively by eNOS in endothelial cells can inhibit apoptosis.

Inflammatory cell apoptosis in atherosclerosis

Recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind plaque growth and development. However, the plaque is dynamic and inflammatory cells are constantly turning over within the core. It is well established that apoptotic cells, particularly macrophages, are present in atherosclerotic plaques in both human and animal models of the disease. Apoptotic macrophages and smooth muscle cells have been identified by TUNEL staining in sections from human plaques by various authors (Bjorkerud & Bjorkerud 1996, Haunstetter & Izumo 1998). Because apoptotic cells are ingested by phagocytes without initiating any further proinflammatory response, it has been suggested that apoptosis may represent a mechanism to regress the plaque. NO is a particularly promising candidate for this strategy because, as well as the pro-apoptotic actions discussed above, it has several other powerful anti-atherogenic characteristics including a powerful inhibitory effect on platelet and inflammatory cell activation (Moncada et al. 1991, Armstrong 2001). Evidence is emerging in support of this hypothesis. For example, administration of L-arginine (the substrate for NOS) to hypercholesterolemic rabbits increases the number of apoptotic macrophages in intimal lesions by three fold. This increase in apoptosis was associated with a regression of the plaque, suggesting that manipulation of the NO synthase pathway may well represent a therapeutic approach to resolving the inflammatory response in the vessel wall (Wang et al. 1999). However, care must by exercised when considering this approach because NO is also known to induce apoptosis in smooth muscle cells (Labelle et al. 2004). Loss of cells from the fibrous cap during the latter stages of atherosclerosis may well be detrimental, destabilising the plaque and promoting rupture (Kockx & Knaapen 2000)

Conclusion

Apoptosis of inflammatory cells is a tightly regulated process whereby cells are removed from the site of inflammation without triggering a subsequent pro-inflammatory response that would instigate further tissue injury. Pharmacological manipulation of apoptosis during chronic inflammatory conditions, such as atherosclerosis, may aid the resolution of inflammation and hence halt, or delay, disease progression. The ubiquitous signalling molecule and inducer of apoptosis, NO, is a likely candidate for such manipulation and may represent a novel therapeutic target for the treatment of such conditions.

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GEA 3162 decomposes to co-generate nitric oxide and superoxide and induces apoptosis in human neutrophils *via* a peroxynitrite-dependent mechanism

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1 GEA 3162 (1,2,3,4,-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-chloride), has powerful effects on neutrophil function and apoptosis, but the underlying mechanisms are unclear, particularly with respect to the possible roles of nitric oxide (NO) and/or peroxynitrite ($ONOO^-$).

2 Our hypothesis was that GEA 3162 is a generator of $ONOO^-$ and that its biological effects on neutrophil apoptosis differ from those of a conventional NO donor. The effects of GEA 3162 were compared to those of the established $ONOO^-$ donor, 3-morpholinosydnonimine (SIN-1), and the NO donor, diethylamine diazeniumdiolate (DEA/NO) in neutrophils from healthy volunteers. Electrochemical detection and electron paramagnetic resonance were used to define the NO-related species generated from these agents.

3 GEA 3162 and SIN-1 influence neutrophil apoptosis differently from DEA/NO. All three compounds induced morphological neutrophil apoptosis. However, both GEA 3162 and SIN-1 paradoxically inhibited internucleosomal DNA fragmentation, whereas DEA/NO induced fragmentation compared to control.

4 In contrast to DEA/NO, generation of free NO was not detectable in solutions of GEA 3162 or SIN-1 (100 μM). However, Cu/Zn superoxide dismutase (SOD; 50–750 U ml^{-1}) unmasked NO generated from these compounds in a concentration-dependent manner. GEA 3162 and SIN-1 oxidised the O_2^-- and $ONOO^-$-sensitive dye, dihydrorhodamine 123 (DHR 123; 1 μM), suggesting that $ONOO^-$ released from these compounds is responsible for oxidation of DHR 123.

5 We conclude that GEA 3162 is an $ONOO^-$ donor with pro-apoptotic properties that more closely resemble SIN-1 than the NO donor, DEA/NO. Moreover, unlike NO, $ONOO^-$ induces apoptosis in neutrophils *via* a mechanism that does not require DNA fragmentation.
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Keywords: Nitric oxide; peroxynitrite; GEA 3162; neutrophil; apoptosis

Abbreviations: DEA/NO, diethylamine diazeniumdiolate; DHR 123, dihydrorhodamine 123; EPR, electron paramagnetic resonance; GEA 3162, 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-chloride; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; IMDM, Iscove's modified Dulbecco's medium; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase

Introduction

Nitric oxide (NO) is a free radical that was originally identified as an endogenous endothelium-derived vasodilator (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987), but is now recognised to have a role in a large number of other physiological and pathophysiological processes, including haemostasis, neurotransmission and inflammation (Quinn *et al.*, 1995). The role of NO in inflammation is particularly complex, with macrophages in particular capable of generating high levels of NO through promotion of transcription factor activity and consequent expression of the unregulated, inducible isoform of NO synthase (iNOS) in response to inflammatory stimuli (Hecker *et al.*, 1996). The primary role of iNOS-derived NO is accepted to be that of a powerful antipathogenic agent, but it is also clear that NO has a complex impact on apoptosis (Dimmeler & Zeiher, 1997; Kim *et al.*, 1999; Taylor *et al.*, 2003), a physiological form of cell death which eliminates effete or unhealthy cells. The issue is further complicated by the rapid reaction of NO with superoxide anion (O_2^-) to form the powerful oxidising agent, peroxynitrite ($ONOO^-$; Kelm *et al.*, 1997).

Despite NO and $ONOO^-$ sharing several biological properties (Ronson *et al.*, 1999; Low *et al.*, 2002), including an ability to induce apoptosis (Taylor *et al.*, 2003), key differences have been noted in their biological effects and mechanisms of action. For example, although both NO and $ONOO^-$ are

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capable of blocking mitochondrial respiration, they do so through inhibition of different complexes in the respiratory chain and their effects are differentially susceptible to reversal by thiols and carbohydrates (Lizasoain *et al.*, 1996). These differences highlight the importance of the accurate identification of the nature of the species generated by the so-called 'NO donors'; $ONOO^-$ generated as a result of concomitant release of NO and O_2^- may exert effects that are similar but mechanistically distinct from pure NO in biological systems. To date, however, little is known about potential differential effects of NO and $ONOO^-$ on inflammatory cell apoptosis.

GEA 3162 is an oxatriazole-5-imine derivative with vasodilator (Nurminen & Vapaatalo, 1996) and pro-apoptotic (Ward *et al.*, 2000; Taylor *et al.*, 2001) properties. However, its identity as a 'pure' NO donor is controversial (Kankaanranta *et al.*, 1996; Holm *et al.*, 1998; Schmidt *et al.*, 2001). In this study, we set out to establish the identity of the NO-related species generated from GEA 3162, and to compare its biological properties to an established NO donor (diethylamine diazeniumdiolate; DEA/NO) and an $ONOO^-$ donor (SIN-1) with respect to neutrophil apoptosis. All the three compounds have previously been shown to accelerate the rate of programmed cell death in neutrophils *in vitro* (Blaylock *et al.*, 1998; Ward *et al.*, 2000; Taylor *et al.*, 2001); however, the precise mechanisms have not been elucidated.

Experimental procedures

Isolation of neutrophils

Neutrophils were isolated from the blood of healthy volunteers as described previously (Ward *et al.*, 1999a). Briefly, whole, citrated blood was centrifuged ($200 \times g$, 20 min) and platelet-rich plasma aspirated. Leukocytes were separated from erythrocytes by dextran sedimentation, then further divided into mononuclear cell and granulocyte populations by centrifugation through a discontinuous Percoll gradient ($720 \times g$, 20 min). Granulocytes were harvested from the 79:68% interface of the gradient, and only neutrophil preparations of $\geqslant 95\%$ purity were used. Neutrophil preparations were tested for cellular homogeneity by preparation of a cytospin slide (100 μl of cell suspension, 300 r.p.m., 3 min), which was then stained and examined by oil-immersion light microscopy, with at least 500 cells counted. The percentage of contaminating leukocytes (eosinophils, monocytes and lymphocytes) was then calculated and the cell preparation was discarded if levels of contamination reached or exceeded 5% of the total cell population.

Apoptosis studies

Neutrophils (4.5×10^6 cells ml^{-1}) were suspended in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley, U.K.) containing penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}), and supplemented with 10% (v v^{-1}) autologous serum. They were cultured in flat-bottomed 96-well Falcon polypropylene plates (37°C, 5% CO_2) for 1–20 h in the presence of either phosphate-buffered saline (PBS) at pH 7.4 (controls) or NO donors (100 μM–3 mM). The concentrations of NO donors used in these studies were selected on the basis of previously published data (Taylor *et al.*, 2001), which have been shown to modulate neutrophil apoptosis.

Following incubation, 100 μl of recovered cells were cytocentrifuged in duplicate, fixed in methanol and stained using Diff-Quik™ physiological stain, then observed by light microscopy ($\times$ 100 objective) to determine the proportion of darkly stained cells with condensed nuclei. At least 500 cells per slide were counted, with the observer blinded to the experimental conditions.

A further 100 μl of recovered cells were centrifuged ($200 \times g$, 3 min), then fixed and permeabilised in 70% ethanol (4°C, 10 min). The cells were washed ($\times$ 3) in PBS without Ca^{2+}/Mg^{2+} before addition of 60 μl RNase A (0.5 mg ml^{-1}) and 60 μl propidium iodide (PI; 0.1 mg ml^{-1}), then assessed by flow cytometry using an EPICS XL2 (Coulter Electronics, Luton, U.K.), to measure DNA fragmentation.

Dihydrorhodamine 123 studies

Dihydrorhodamine 123 (DHR 123) is a fluorescent dye which is activated by various reactive oxygen species, including $ONOO^-$, O_2^-, hydrogen peroxide (H_2O_2) and HOCl, but not by NO (Crow, 1997). This compound can therefore be used to discriminate between agents that release NO only and those that generate NO and O_2^- simultaneously.

PBS (100 μl), the neutrophil-activating agent, phorbol 12-myristate 13-acetate (PMA; final concentration 10 nM), SIN-1 (1 mM), GEA 3162 (100 μM) or DEA/NO (1 mM) was added to 900 μl PBS in 2 ml Eppendorf tubes. DHR 123 was added to each tube to a final concentration of 1 μM. Tubes were incubated for 60 min (37°C, 5% CO_2). A volume of 450 μl was then transferred to a 0.5 ml cuvette, excited at 480 nm and the fluorescence emitted at 500 nm was read using a spectrofluorimeter (Perkin Elmer, U.K.).

Electrochemical detection of NO

Free NO from GEA 3162 or SIN-1 (both 100 μM) and DEA/NO (5 μM) was measured in Iscove's MDM cell culture medium using an NO electrode (Iso-NO II, World Precision Instruments) calibrated daily with DEA/NO (0.1–1.6 μM) in pH 4 buffer. Superoxide dismutase (SOD; 50–750 U ml^{-1}) was added cumulatively in an effort to unmask NO generated from these agents (Lizasoain *et al.*, 1996), and the concentration of NO was measured at plateau approx. 5 min following SOD addition.

Electron paramagnetic detection of O_2^- and $ONOO^-$

Spin-trapping experiments were performed in Iscove's MDM cell culture medium containing 100 μM SIN-1, GEA 3162 or DEA/NO in the presence or absence of SOD (500 U ml^{-1}), and the spin trap Tempone-H (1 mM) (Dikalov *et al.*, 1997) prepared in water containing EDTA (10 mM). Reaction mixtures were incubated at 37°C throughout the experiments and the intensity of the electron paramagnetic resonance (EPR) signal corresponding to the formation of 4-oxo-Tempo (triplet centred at 3364 G) was measured (arbitrary units) at timed intervals for each of the donor drugs (Magnettech® miniscope MS100 with the following parameter settings: field

sweep 51.2 G, microwave frequency 9.5 GHz, microwave power 20 mW, modulation amplitude 1500 mG). In control experiments without any NO-generating compounds present, there was a slow increase in EPR signal corresponding to the auto-oxidation of Tempone-H to 4-Oxo-Tempo; these signals have been subtracted from the data shown.

Statistical analysis

Data were assessed for statistical significance using two-way analysis of variance (ANOVA) with a Student–Neuman–Keuls post-test. Probability values of $P<0.05$ were considered statistically significant.

Results

Electrochemical detection of DEA/NO-derived NO revealed that this compound liberated free NO in a spontaneous and predictable manner. A bolus addition of DEA/NO (5 μM) to culture medium caused a rapid transient rise in NO concentration, reaching a maximum of $\sim 5\,\mu$M, before subsiding over the subsequent 30 min (Figure 1a). The signal was not altered by incubation with SOD (150 U ml^{-1}), but the rate of decay of the NO signal was markedly enhanced in the presence of neutrophils (5×10^6 ml^{-1}; Figure 1a). SOD (150 U ml^{-1}) failed to increase the signal seen with neutrophils; instead, it caused a significant and surprising inhibition of NO generation.

In contrast to DEA/NO, NO was not detectable from GEA 3162 (100 μM) in medium. However, NO generation from this agent was detected in the presence of SOD (150 U ml^{-1}), with maximal NO concentrations reaching $\sim 3\,\mu$M after 30 min incubations (Figure 1b). In the presence of neutrophils (5×10^6 ml^{-1}) and SOD (150 U ml^{-1}), NO generation was markedly attenuated for incubation periods of >10 min. NO generation bore a nonlinear relationship to SOD concentration for both GEA 3162 and SIN-1 (Figure 1c), and there was no significant difference between the two agents under these conditions ($P>0.05$).

Results from EPR spectroscopy identified a signal corresponding to 4-oxo-Tempo formation from the reduced form of the spin-trap, Tempone-H, after incubation with GEA 3162 or SIN-1 (Figure 2a). The intensity of the signal obtained with both GEA-3162 and SIN-1 was dependent on the incubation time and was severely attenuated in the presence of SOD (500 U ml^{-1}). DEA/NO failed to generate a significant signal under the same conditions (Figure 2b).

Assessment of the oxidation of DHR 123 to fluorescent rhodamine 123 using a spectrofluorimeter showed that both SIN-1 (1 mM) and GEA 3162 (100 μM) produced high levels of fluorescence (803 $\pm$ 74 and 757 $\pm$ 100 U, respectively; $P<0.05$; $n=3$) compared to control (PBS alone, 23 $\pm$ 5 U; Figure 3). In contrast, DEA/NO failed to significantly oxidise DHR 123 (53 $\pm$ 9 U; $n=3$). None of the compounds alone (in the absence of DHR 123) produced a fluorescent signal that exceeded 1.0 arbitrary units (data not shown), thus excluding autofluorescence as a possible explanation for the differential fluorescence observed.

High concentrations of DEA/NO (1 mM) and GEA 3162 (100 μM) accelerated morphological changes characteristic of

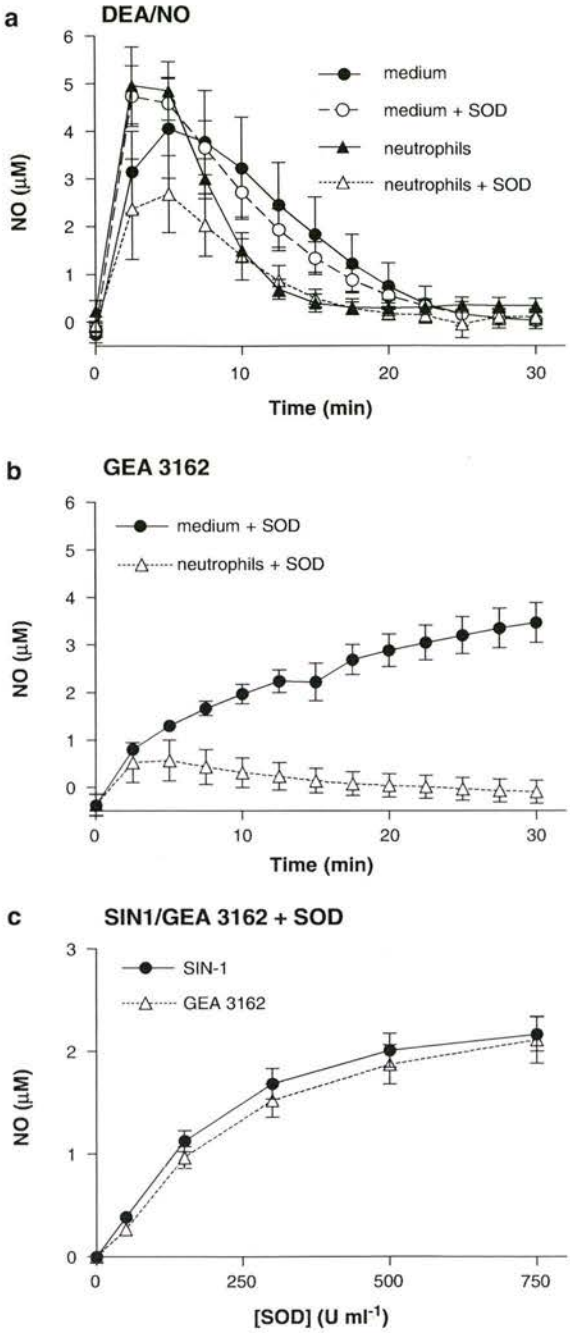

Figure 1 Measurement of NO release from DEA/NO, SIN-1 and GEA 3162 in the absence and presence of neutrophils and/or SOD. Mean NO electrode recordings for (a) DEA/NO (5 μM) and (b) GEA-3162 (100 μM) in Iscove's medium, in the presence and absence of human neutrophils (4.5×10^6 cells ml^{-1}). NO release from GEA 3162 was not detectable in the absence of SOD (150 U ml^{-1}). Concentrations of NO were calculated using a calibration curve generated using DEA/NO (100 nM 1.6 μM) in pH 4 buffer. Results are mean $\pm$ s.e.m. ($n=6$). (c) Generation of NO by SIN-1 and GEA 3162 (both 100 μM) in the presence of SOD (50–750 U ml^{-1}). Data represent NO concentrations at plateau approx. 5 min following addition of SOD. Results are mean $\pm$ s.e.m. ($n=6$).

neutrophil apoptosis over a 20-h time course compared to untreated controls (Figure 4a). Approximately 50–60% of control cells underwent spontaneous apoptosis during the

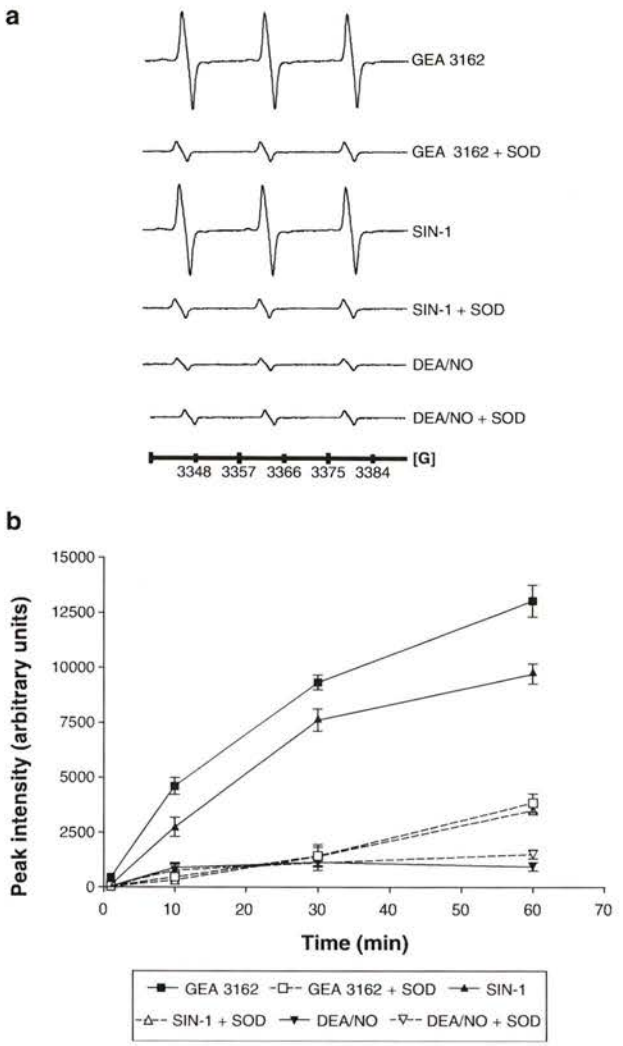

Figure 2 Generation of O_2^-/$ONOO^-$ by donor drugs. (a) Representative EPR spectra showing the signal generated through oxidation of Tempone-H (1 mM) by O_2^-/$ONOO^-$ after incubation (30 min, 37°C) with SIN-1, GEA 3162 and DEA/NO (all 100 μM) in the presence or absence of SOD (500 U ml^{-1}). Control spectra of Iscove's medium and 1 mM Tempone-H have been subtracted from each trace. (b) Relative intensities (arbitrary units) for EPR signals generated over time in the presence of SIN-1, GEA-3162 or DEA/NO (100 μM) in the presence or absence of SOD (500 U ml^{-1}). Results are mean ± s.e.m. ($n=6$).

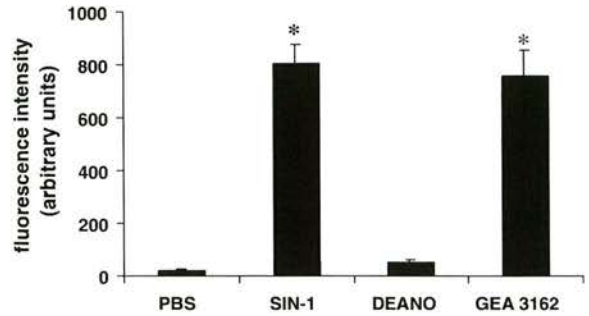

Figure 3 Spectrofluorimetric assessment of DHR 123 oxidation. DHR 123 (1 μM) was added to solutions of PBS (control), SIN-1 (1 mM), DEA/NO (1 mM) or GEA 3162 (100 μM) and incubated for 60 min at 37°C. The extent of DHR 123 oxidation to rhodamine 123 was assessed using a spectrofluorimeter at an emission wavelength of 500 nm. Results are mean ± s.e.m. ($n=3$). Asterisks represent significant difference ($P<0.05$) from control fluorescence.

culture period, whereas those treated with either compound were virtually all apoptotic. However, when internucleosomal DNA fragmentation was assessed by flow cytometric measurement of PI intercalation, key differences were observed between the two compounds. Whereas DEA/NO increased DNA fragmentation compared to control, GEA 3162 produced an unexpected inhibition of this process (Figure 4b). The effects of 1–3 mM SIN-1 on morphology and DNA fragmentation at 20 h were then studied in order to determine how $ONOO^-$ influences these apoptotic events in neutrophils. This compound had effects similar to GEA 3162 but opposing effects to DEA/NO, in that a concentration-dependent inhibition of DNA fragmentation occurred alongside an induction of apoptotic morphology (Figure 4c).

Discussion

Our results show that the oxatriazole-5-imine-derived compound, GEA 3162, generated an oxidising species that induced apoptosis in human neutrophils *via* a mechanism that was independent of DNA fragmentation. Similar results were obtained with the known $ONOO^-$ generator, SIN-1, but the NO donor, DEA/NO, failed to generate an oxidising species, and apoptosis induction in response to this agent was associated with increased DNA fragmentation. Further experiments to determine the NO-related species generated from these agents established that GEA 3162 and SIN-1 concomitantly generate NO with O_2^-, resulting in rapid formation of $ONOO^-$. The NO component was unmasked by removal of O_2^- by high concentrations of the enzyme, SOD. These results were in common with those of the recognised $ONOO^-$ generator, SIN-1 (Hogg *et al.*, 1992), but were at odds with the NO donor drug, DEA/NO (Maragos *et al.*, 1991), which was found to generate NO in the absence of O_2^- scavenger systems, and did not oxidise Tempone-H to generate an EPR signal.

Previous studies have suggested that GEA 3162 does not liberate O_2^- alongside NO; therefore it was suggested to be a 'pure' NO donor (Holm *et al.*, 1998). However, in contrast, recent preliminary data have indicated that GEA 3162 resembles SIN-1, in that both compounds simultaneously generate NO and O_2^- and are therefore both $ONOO^-$ donors (Schmidt *et al.*, 2001). Given that SIN-1 is accepted to be an $ONOO^-$ donor (Hogg *et al.*, 1992), while DEA/NO generates pure NO (Maragos *et al.*, 1991), our results suggest that $ONOO^-$ is a requirement for the inhibition of DNA fragmentation and implies that GEA 3162 is also an $ONOO^-$-generating agent. We therefore carried out a number of experiments to determine the nature of the NO-related species liberated from GEA 3162.

An NO-specific electrode detected NO release from DEA/NO in culture medium, as has been shown previously (Crane *et al.*, 2002). Release of NO from this compound (5 μM) occurred spontaneously at physiological temperature and pH. The signal was not significantly affected by SOD alone, suggesting that there was no concurrent release of O_2^- under these conditions. In the presence of neutrophils, there was a mild acceleration of the decay of NO signal from DEA/NO,

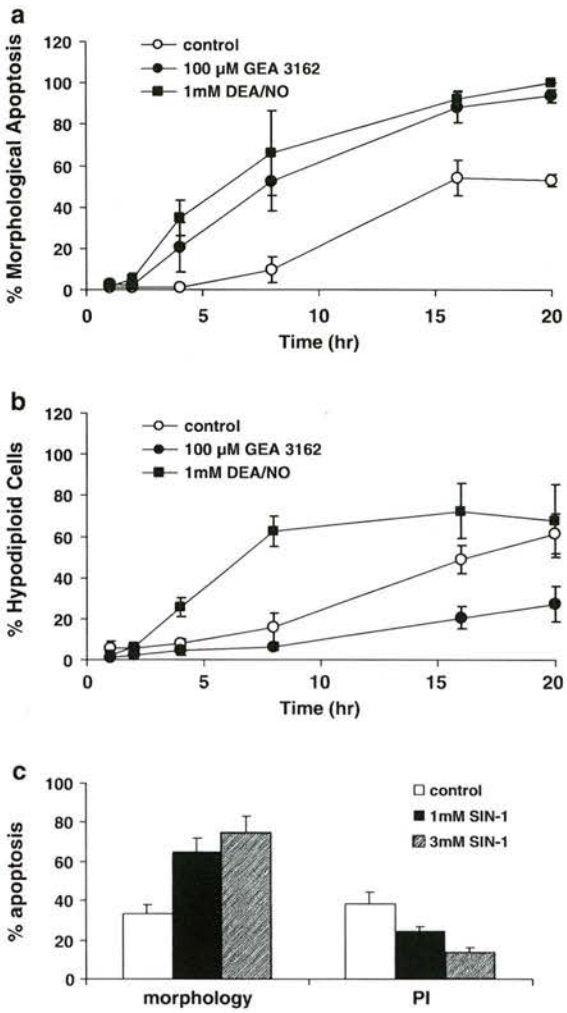

Figure 4 Differential effects of compounds on independent parameters of neutrophil apoptosis. Human neutrophils were incubated at 37°C for 1–20 h with PBS (control), DEA/NO (1 mM) or GEA 3162 (100 μM). Apoptosis was then assessed by (a) morphological changes using oil-immersion light microscopy and (b) PI intercalation to show hypodiploid DNA content characteristic of apoptotic cells. Results are mean ± s.e.m. ($n = 3$–6). (c) Neutrophils were incubated for 20 h with SIN-1 (1–3 mM) and apoptosis was assessed by morphology and PI intercalation. Results are mean ± s.e.m. ($n = 5$).

suggesting a scavenging effect of the cells. However, NO detection in the presence of neutrophils and SOD was not enhanced; indeed, it was surprisingly blunted, perhaps indicating a scavenging effect of the enzyme under these conditions. On the other hand, SIN-1 and GEA 3162 failed to produce measurable free NO under the same conditions, despite the relatively high concentrations of these drugs used (100 μM). The limit for NO detection of the electrode used in these studies is ~50 nM. Rapid scavenging of NO through reaction with O_2^- generated simultaneously might account for the lack of detectable NO under these conditions. It is well established that this is the case for SIN-1, which is recognised to be an $ONOO^-$ donor rather than a pure NO donor (Hogg *et al.*, 1992), but it was a matter of debate whether NO release from GEA 3162 also involves the concomitant liberation of O_2^-. In order to test this, the buffer into which the electrode was immersed was preincubated with varying concentrations of Cu/Zn SOD, which rapidly converts O_2^- to H_2O_2, in an effort to 'unmask' NO by removal of O_2^-. If SIN-1 and GEA 3162 both liberate a combination of NO and O_2^-, then free NO might be detectable in the presence of SOD, which would prevent at least some of the NO being oxidised to $ONOO^-$ (Lizasoain *et al.*, 1996). With reference to the rate constants, the relative rates of reaction can be estimated as follows (for a concentration of 1 μM NO and assuming the amount of NO generated is matched by O_2^-):

$$2O_2^- + H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad k = 2\times10^9 M^{-1}s^{-1} \quad \text{Rate} = 2\mu Ms^{-1}$$

$$O_2^- + NO \longrightarrow ONOO^- \quad k = 6\times10^9 M^{-1}s^{-1} \quad \text{Rate} = 6\mu Ms^{-1}$$

From these reaction rates, it is reasonable to predict that some NO might be unmasked in the presence of SOD, but that the amount observed would be a relatively small percentage of the total amount generated, on account of the fact that the rate of reaction of NO with O_2^- is three times faster than that catalysed by SOD. In the event, NO was detected from both SIN-1 and GEA 3162; indeed, the profile of NO release from the two compounds in the presence of SOD was virtually identical, and correlated well with previously published data for SIN-1 (Lizasoain *et al.*, 1996). The role of oxygen-derived free radicals in quenching of NO was confirmed by EPR experiments using the O_2^- and $ONOO^-$-specific spin-trap, Tempone-H. A signal corresponding to 4-oxo-Tempo increased in intensity during incubations with both SIN-1 and GEA-3162, but was not seen with DEA/NO. The 4-oxo-Tempo signal was quenched by SOD, indicating that the species responsible for oxidation of Tempone-H was O_2^-, or a downstream product of O_2^- (e.g. $ONOO^-$). Taken together, these results indicate that GEA-3162 is indeed an $ONOO^-$ generator, with characteristics that are very similar to SIN-1 and quite distinct from the NO donor, DEA/NO.

DHR 123 is oxidised to the fluorochrome, rhodamine 123, by a number of oxidative and nitrosative species, including $ONOO^-$, O_2^-, H_2O_2 and HOCl, and is widely used in flow cytometric assessment of respiratory burst in neutrophils (Smith & Weidemann, 1993; Ruchaud-Sparagano *et al.*, 1997). However, it has been demonstrated that NO does not have the capacity to oxidise this molecule (Crow, 1997). This makes DHR 123 an ideal tool for discriminating between agents that release NO and those that liberate $ONOO^-$. Addition of DHR 123 to solutions of SIN-1 (1 mM) and GEA 3162 (100 μM) led to the generation of fluorescent rhodamine 123, whereas with PBS and DEA/NO (1 mM), no fluorescence could be detected. This suggests that both SIN-1 and GEA 3162 generate species distinct from NO, such as $ONOO^-$, which does react with DHR 123.

Functionally, SIN-1 and GEA 3162 share properties that differ from DEA/NO. Apoptosis studies showed that all the three compounds accelerated morphological neutrophil apoptosis at high concentrations, as has previously been demonstrated by this group (Ward *et al.*, 2000; Taylor *et al.*, 2001). Only extremely high concentrations of DEA/NO were able to induce neutrophil apoptosis (Taylor *et al.*, 2001), which are likely to be suprapathophysiological, thereby suggesting that even high concentrations of NO generated from iNOS during excessive inflammation are unable to promote neutrophil apoptosis by themselves. Therefore, endogenously formed NO,

whatever its source, is unlikely to induce apoptosis in this cell type if it is not converted to $ONOO^-$.

Only DEA/NO produced the expected rise in the level of internucleosomal DNA fragmentation that mirrored the morphological changes observed. In the presence of both SIN-1 and GEA 3162, this process was inhibited compared to control, indicating an uncoupling of DNA fragmentation from other apoptotic events with these two compounds, and provides further evidence that the species generated by GEA 3162 more closely resembles that from SIN-1 than from DEA/NO. Although this study did not investigate the potential underlying mechanism, this phenomenon could potentially be due to the inhibitory modification of tyrosine (nitration) or reduced cysteine (*S*-nitrosation) residues (Kuo & Kocis, 2002) in critical proteins of the DNA fragmentation pathway such as caspase 3 (Kim *et al.*, 1997) or DFF40 (Widlak, 2000), thus affecting the activity of these proteins and therefore the DNA fragmentation process.

Overall, we have demonstrated that GEA 3162 is an $ONOO^-$ donor that induces neutrophil apoptosis *via* a mechanism that is not dependent on DNA fragmentation. Chemically and biologically, therefore, GEA 3162 resembles SIN-1 and is clearly distinct from DEA/NO. The induction of neutrophil apoptosis may be of therapeutic benefit in a number of inflammatory conditions in which resolution of inflammation is impaired (such as arthritis, pancreatitis, pneumonia and asthma), which causes neutrophils to persist in the tissue and then potentially subsequently die by necrosis (Ward *et al.*, 1999b; Taylor *et al.*, 2003). $ONOO^-$ is an important molecule in inflammation as iNOS-derived NO and O_2^- are both produced by inflammatory cells and the resulting $ONOO^-$ may contribute to the pathophysiology of a number of chronic inflammatory conditions (Szabo, 1996).

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Apoptosis and Atherosclerosis: The Role of Nitric Oxide

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Abstract: Atherosclerosis, and its associated complications, are a major cause of morbidity and mortality, and it is now recognised as a chronic inflammatory disorder. Progression of inflammation depends on the balance between recruitment of inflammatory cells and their subsequent removal from a site of inflammation. Apoptosis, or programmed cell death, is a fundamental process governing cell survival and is a major determinant of the resolution of the inflammatory response. Apoptotic cells are instantly recognised for non-inflammatory clearance by phagocytes (e.g. macrophages) and removed from the vicinity of inflammation without the release of their pro-inflammatory cell contents. Nitric oxide (NO) plays an important role in many biological processes and has several anti-atherogenic properties including vasodilatation, inhibition of platelet activation and aggregation, and the regulation of apoptosis in a variety of cell types involved in atherogenesis. A critical early event during atherogenesis is injury to the endothelium. The ensuing damage results in endothelial dysfunction, including a reduction in the capacity of the endothelium to generate NO. Decreased NO bioavailability is likely to influence many cellular processes occurring within atherosclerotic lesions, including apoptosis. Modulation of apoptosis is a novel target for therapeutic intervention in the treatment of chronic inflammatory disorders, such as atherosclerosis. This modulation may help limit or resolve inflammation without the concomitant recruitment of subsequent inflammatory cells, thereby reducing the potential for further tissue damage. NO is a possible candidate for manipulation of atherosclerotic processes due to both its powerful anti-atherogenic characteristics and ability to affect apoptosis. This review highlights the role of apoptosis in atherosclerosis and discusses the therapeutic potential of NO to limit and/or resolve vascular inflammation.

Keywords: Atherosclerosis, nitric oxide, inflammation

INTRODUCTION

Atherosclerosis and its associated secondary complications, such as myocardial infarction and stroke, remain a major cause of morbidity and mortality in industrialised nations. Atherosclerosis is a multifactorial disease process with a complicated aetiology. However, it is now widely recognised that there is a chronic inflammatory component to the disease process, which is characterised by the formation of lipid-rich plaques in the wall of major conduit vessels such as the coronary arteries and aorta [1-4]. These lesions are usually eccentric and made up of a necrotic core of lipid-laden inflammatory cells encapsulated by a fibrous, collagen-rich cap consisting of vascular smooth muscle cells (VSMC) and extracellular matrix [5]. In this state, the plaque is considered 'stable'; its physical presence results in partial occlusion of the vessel, but because the vessel is dynamic rather than static, it can often compensate for this occlusion and accommodate for the presence of the plaque without a decrease in lumen diameter [6]. However, if the cap is subject to mechanical breakdown or erosion, the plaque can becomes 'unstable' and ruptures. When the plaque cap is compromised, the highly thrombogenic contents of the core are released into the circulation. Platelets are rapidly recruited and activated resulting in thrombus formation. Plaque rupture can occur several times and remain sub-clinical with the VSMC cap reforming, or healing, over the top of the thrombus which becomes incorporated into the lesion, resulting in a layering effect within the plaque [7]. This process can further occlude the vessel *in situ,* or the thrombus can detach from the plaque surface, and the resulting embolus occlude smaller vessels downstream, leading to the more serious acute cardiovascular syndromes, such as myocardial infarction and stroke [8]. The determinants of plaque vulnerability to destabilisation and rupture have yet to be fully identified, but a growing body of evidence is emerging that points toward a critical role for both the thickness of the VSMC layer overlaying the core [9] and to inflammatory processes occurring within the plaque [10-12].

APOPTOSIS

Inflammatory cell apoptosis, or programmed cell death, is a highly regulated process whereby cellular death and subsequent phagocytosis occur without disruption of the cell membrane and ensuing release of the histotoxic and pro-inflammatory mediators from the cytoplasm [13-15]. Apoptosis therefore represents a non-inflammatory mechanism for the removal of cells from a site of tissue damage, and hence, is critical to the successful resolution of the inflammatory response. Pharmacological manipulation of apoptosis in a variety of cell types, particularly inflammatory cells, may represent a novel therapeutic strategy for the treatment of chronic inflammatory disorders, including atherosclerosis [16, 17].

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Apoptosis can be modulated by the endogenous messenger nitric oxide (NO), which can be both pro- and anti-apoptotic depending on local concentration, the specific cell type in question and the NO-related species generated *in vivo* [18, 19]. The aim of this review is to examine the role of apoptosis in atherosclerosis and to discuss the potential of this process as a target for therapeutic intervention by NO donor drugs, whereby disease progression may be stabilised in an effort to prevent the acute cardiovascular syndromes.

ATHEROSCLEROSIS

Despite arduous research in both humans and animal models of the process, the underlying causes of atherogenesis remain largely unknown. It is widely accepted that a critical early event of atheroma development is endothelial cell injury and damage. This can occur by several mechanisms: chemical injury can occur through oxidative stress, including oxidation of circulating low density lipids (LDL) resulting in the formation of damaging oxidised LDL (ox-LDL); and physical damage to the endothelium can result from shear stress within the vessel, with plaques tending to form at sites usually subjected to elevated shear stress, such as bifurcation points in the arterial tree or where low shear stress results in reduced stimulation of NO synthase and NO generation [20]. The consequences of an insult to the endothelium are numrous fold: firstly, the damaged endothelium becomes dysfunctional and the net production of NO by eNOS decreases, promoting vasoconstriction. This is reflected in patients with risk factors for atherosclerosis, such as hypercholesterolaemia. In hypercholesterolaemic individuals, vasodilatation in response to the endothelium-dependent vasodilator, acetylcholine (which acts by triggering NO release from the endothelium), is impaired in forearm resistance vessels compared to normocholesterolaemic controls. However, vasodilatation in response to the endothelium-independent NO donor, sodium nitroprusside (SNP), is unaffected. This suggests that endothelial injury resulting from the hypercholesterolaemic state decreases the normal NO-producing capacity of the endothelium [21]. Although resistance vessels are not usually susceptible to plaque formation, they are widely accepted to reflect changes occurring globally throughout the arterial tree.

In addition, in response to the insult to the endothelium, an inflammatory response is triggered. Depending on the nature and duration of the insult, this response can become excessive and, over a period of years, constitutes the disease process itself [2, 3]. The inflammatory process begins with the expression of chemotactic and adhesion molecules for monocytes and lymphocytes, including vascular cell adhesion molecule 1 (VCAM-1), on the surface of dysfunctional endothelial cells. Circulating monocytes adhere to the site of endothelial damage and translocate to the sub-endothelial space where they accumulate [22]. Colony stimulating factors secreted from areas of endothelial damage induce monocytes to differentiate into macrophages, which then begin to express scavenger receptors, facilitating the internalisation of oxidised low density lipoprotein (ox-LDL) [23]. Cultured macrophages do not accumulate native LDL on account of downregulation of LDL receptors in the cholesterol-replete state. However, once LDL has been modified by oxidation, accumulation of the resultant ox-LDL by macrophages continues unchecked because scavenger receptors are not downregulated by cholesterol accumulation. The mechanism of lipid peroxidation is still not fully understood, but free radicals such as superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($^{\cdot}OH$), undoubtedly have a role to play [24, 25]. Peroxynitrite ($ONOO^-$), formed by the rapid reaction of NO with $O_2^{\cdot-}$, can also initiate lipid peroxidation, both *in vitro,* and in membrane lipids and lipoproteins [26, 27]. In the lipid-laden condition, macrophages are known as foam cells and it is an accumulation of these cells in the vessel intima which forms the earliest recognisable lesion of atherosclerosis – the fatty streak (Fig. **1A**) [20, 28]. Fatty streaks have been observed in the vessels of young adults and children (including neonates), suggesting that atherosclerosis may be initiated early in life but remains sub-clinical unless vessel occlusion and/or plaque rupture lead to diagnosis [29].

Once the fatty streak is established, the plaque grows in size as the ox-LDL accumulated in macrophage-derived foam cells causes both further endothelial damage and is chemoattractant for circulating monocytes, which freely migrate through the endothelium, accumulate in the sub-endothelial space, and differentiate into macrophages which then go on to become foam cells [30]. This establishes a perpetual cycle of endothelial damage, leading to monocyte recruitment and accumulation of ox-LDL, which encourages further endothelial damage (Fig. **1**). Activated macrophages in fatty streaks secrete numerous cytokines and growth factors including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) [20]. All of these factors and cytokines induce vascular smooth muscle cell (VSMC) hypertrophy and/or hyperplasia. This represents a change in phenotype for VSMCs, from the usual adult vascular contractile phenotype to the synthetic phenotype usually seen only in developing vessels. However, characterisation of VSMCs into only two distinct phenotypes is a vast over-simplification. A wide spectrum of diverse intermediary phenotypes exist under different physiological and pathophysiological conditions, without a clear distinction between phenotypes. This is especially true when VSMCs are undergoing phenotypic transition and there may, in fact, be several sub-populations of VSMCs present within the plaque at any given time [31]. In a synthetic phenotype, VSMCs have the ability to proliferate and synthesise large amounts of collagen. Proliferation begins in the media of the vessel, but after a time the cells begin to migrate, invade the intima and form a layer over the top of the fatty streak. This layer of VSMCs secretes large amounts of extracellular matrix and connective tissue consisting of glycosaminoglycans, dermatan sulfate, and collagen. These molecules form a mesh over the fatty streak, which becomes calcified and forms the fibrous cap of the plaque, encapsulating the highly thrombogenic lipid core and maintaining a barrier between the plaque contents and the circulation (Fig. **1A**) [4, 5].

APOPTOSIS IN ATHEROSCLEROSIS

Inflammatory Cells

Recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind

plaque growth and development. Apoptosis of inflammatory cells within an atherosclerotic lesion may result in their subsequent phagocytosis and removal from the plaque core without the concomitant recruitment of additional inflammatory cells (Fig. **1B**). The identity of the cells responsible for clearing the apoptotic macrophages from the plaque remains to be confirmed. It is currently unclear whether activated macrophages can themselves phagocytose populations of apoptotic inflammatory cells, or if this is done by a separate subset of specialised phagocytes.

Apoptotic macrophages have been located by TUNEL staining in plaques from animal models of atherosclerosis and in plaques excised from human vessels [32-34]. The apoptotic macrophages tend to be clustered in areas of the plaque most vulnerable to rupture, most notably the base, or shoulder, regions where the VSMC prevalence is also decreased [35]. It is currently unclear why these areas of the plaque are particularly susceptible to rupture, however, reduced VSMC localisation in these areas of vulnerable plaques may indicate that apoptosis of a variety of cell types, including VSMCs, may play a role in plaque susceptibility to rupture (Fig. **1C**) [36]. Recently, it has been suggested that the macrophage-enzyme, myeloperoxidase (MPO), could also have an important role in determining plaque vulnerability. MPO, a member of the heme peroxidase superfamily, generates reactive oxidants including hypochlorous acid (HOCl) as part of its normal function in innate host defences [37, 38]. Sugiyama *et al.* have described a strong co-localisation between macrophage MPO expression and HOCl-modified proteins at sites of lesion rupture in patients who suffer acute cardiac events [39]. MPO-generated HOCl at relatively high physiological concentrations, but still within the range expected to be produced at areas of vascular inflammation (30 – 50 μM), has been shown to promote endothelial cell death by stimulating apoptotic pathways including rapid caspase-3 activation and DNA fragmentation [40]. This observation suggests that, prior to undergoing apoptosis themselves, activated macrophages may induce apoptosis of neighbouring endothelial cells through MPO expression, resulting ultimately in plaque rupture.

Vascular Smooth Muscle Cells

As previously mentioned, loss of VSMCs from the protective plaque cap is a major determinant of plaque rupture. Because healthy endothelial cells secrete factors that promote VSMC survival, a consequence of activated macro-

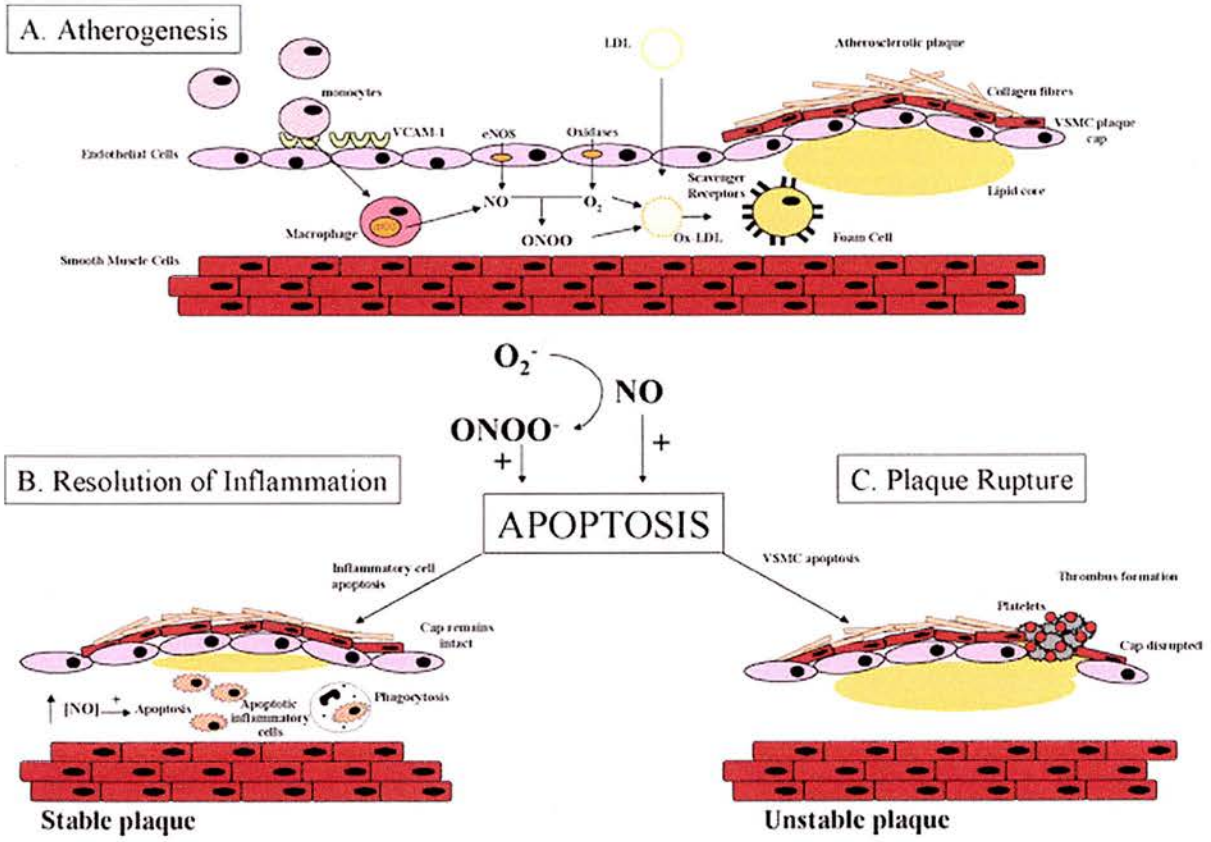

Fig. (1). During atherogenesis circulating monocyes translocate to the subendothelial space where they accumulate modified lipids and form an atherosclerotic plaque, overlain with a fibrous cap of VSMCs (A). Apoptosis can be induced in a variety of cell types by NO. Apoptotic inflammatory cells are cleared by phagocytes, aiding resolution of the inflammatory response, and ultimately facilitating plaque regression (B). However, apoptosis of VSMC may be detrimental, resulting in degradation of the plaque cap and leading to plaque rupture, particularly in the shoulder regions, and ultimately to thrombus formation (C).

phage-induced endothelial cell death is an increase in VSMC death [41]. Direct induction of VSMC apoptosis in the apolipoprotein E null murine model of atherosclerosis induces both rupture and thrombosis of the plaque [42]. In addition to removing the protective presence of the endothelium, macrophages can also influence VSMC apoptosis directly. Activated human monocytes/macrophages have been found to be responsible for the death of human VSMCs *in vitro* [43, 44]. Seshiah *et al.* have hypothesised that macrophage colony-stimulating factor (M-CSF), a haematopoietic growth factor supporting survival and differentiation of monocytes, is secreted from VSMCs resulting in macrophage activation, ultimately triggering subsequent VSMC apoptosis [44]. The exact mechanism of this process remains to be fully understood but is thought that macrophages prime VSMCs to respond to apoptotic stimuli, for example by triggering the expression of death receptor ligands such as TNFα on the cell surface [45].

NITRIC OXIDE

NO was first identified as the biological messenger responsible for endothelium dependent vasodilatation [46, 47]. Since then, it has become increasingly clear that NO has many diverse actions beyond that of control of vascular tone. Synthesised by three isoforms of the enzyme NO synthase (NOS), NO is now known to inhibit platelet and inflammatory cell adhesion and activation, to act as a transmitter at non-adrenergic non-cholinergic (NANC) neurones, and to exhibit both pro- and anti-apoptotic effects depending on the NO-related species, concentration and cell type in question [48].

The classical pathway by which NO exerts its effects is *via* activation of the enzyme soluble guanylate cyclase (sGC) and resultant conversion of guanosine 5'-triphosphate (GTP) to 3', 5'-cyclic guanosine monophosphate (cGMP) [48, 49]. However, recent studies have established that NO can act *via*, as yet unidentified, cGMP-independent pathways in various systems, particularly during the inhibition of platelet aggregation [50-53] and regulation of inflammatory cell apoptosis [51-54].

There is currently much debate as to whether all of the effects of NO are mediated by the NO radical *per se*, or by other NO-related species formed *in situ.* Due to its free radical status, NO is highly reactive, and combines readily with reactive oxygen species (ROS) to form a variety of NO-related species. For example, superoxide anions (O_2^-), often generated at sites of inflammation by the inflammatory cell enzyme NADPH oxidase [55], react rapidly with NO to produce toxic peroxynitrite ($ONOO^-$). This reaction is usually prevented *in vivo* by the battery of antioxidants that inactivate ROS. For instance, the enzyme superoxide dismutase (SOD) removes O_2^- by catalysing its conversion to hydrogen peroxide (H_2O_2), which is subsequently further inactivated by catalase [56]. However, if the production of ROS is such that the antioxidant capacity is overcome, then any free NO present will react rapidly with the O^-_2 to form $ONOO^-$. NO can also form S-nitrosothiols *via* the transfer of NO^+ from higher oxides of nitrogen (e.g. N_2O_3) to reduced thiol groups on proteins such as albumin, resulting in the formation of S-nitrosoalbumin [57, 58]. It has recently been hypothesised that the formation of S-nitrosothiols may act as a slow-release NO store *in vivo* [50]. S-nitrosation of a range of cellular proteins is now considered to be one of a number of post-translational modifications that can alter protein function, and these modifactions are likely to be responsible for many of the cGMP-independent effects of NO, including inhibition of caspase-3 activation [59, 60].

NO AS A MEDIATOR OF APOPTOSIS

The pro- and anti-apoptotic actions of NO have been well documented in many cell systems. Current evidence suggests that lower concentrations of NO produced constitutively by endothelial and neuronal NOS (eNOS and nNOS) are cytoprotective *via* primarily cGMP-dependent mechanisms, whilst higher, supraphysiological concentrations generated in some pathologies by inducible NOS (iNOS) mediate apoptosis *via* mechanisms independent of cGMP signalling [61]. For example, high concentrations of either exogenous or endogenous iNOS-derived NO have been shown to induce apoptosis in murine and rabbit macrophage cell lines [62, 63]. Apoptosis in rabbit macrophages was unaffected by inhibition of cGMP-dependent kinases and not mimicked by cGMP analogs, suggesting that the pro-apoptotic actions of NO are cGMP-independent [64]. Conversely, pre-treatment with relatively low concentrations of exogenous NO, delivered by the synthetic NO donor sodium nitroprusside (SNP) protects RAW 264 cells (a mouse macrophage cell line) against cell death upon subsequent exposure to higher concentrations of NO which would normally be cytotoxic [65]. Furthermore, this protection was mimicked by cGMP analogs but negated in the presence of sGC inhibitors, suggesting that the cellular protection was conferred by cGMP. Similarly, high levels of NO produced by activated macrophages as a consequence of iNOS upregulation may also induce VSMC apoptosis through DNA damage and subsequent p53 activation [66].

This dual role, and apparent paradox of NO, may be explained, at least in part, by the free radical nature of NO and the ease with which it will form various NO-related species *in vivo*. As already mentioned, NO will react rapidly with O_2^- to form $ONOO^-$. The production of $ONOO^-$ may be of particular importance at sites of vascular inflammation such as atherosclerotic lesions, where the concentration of reactive oxygen is likely to be elevated [67]. The precise role of $ONOO^-$ in inflammatory cell apoptosis remains to be elucidated. There is some evidence to suggest that at high concentrations (100 μM – 300 μM), $ONOO^-$ induces apoptosis in RAW 264.7 cells [68], whilst at lower concentrations (30 μM – 50 μM), it may have a protective effect against lipopolysaccharide (LPS) and interferon (IFN)-γ-induced apoptosis in these cells [69]. Brockhaus *et al.* [70] have demonstrated that overexpression of copper/zinc SOD (CuZnSOD) can protect RAW 264.7 cells against apoptosis initiated by NO, either exogenous or iNOS-derived. This implies a role for $ONOO^-$ as the mediator of NO-initiated apoptosis. However, whilst a specific scavenger of $ONOO^-$, uric acid, effectively abolishes apoptosis induced by the $ONOO^-$ generator SIN-1, it left apoptosis induced by the NO donors S-nitrosoglutathione (GSNO) and spermine diazeniumdiolate SPER/NO unaltered, suggesting that NO and NO-related species

may be able to activate several pathways when initiating apoptosis [70].

Whilst endogenous macrophage iNOS-derived NO has been shown to induce apoptosis in animal models, this is not necessarily the case in human macrophages, which might not produce any NO at all. [71-73]. However, despite this reduced capability to produce endogenous NO, human macrophages still undergo apoptosis in response to exogenous NO and NO-related species. For example, the NO donors GSNO and SPER/NO induce apoptosis in primary human monocyte-derived macrophages [74].

NO AND APOPTOSIS IN THE RESOLUTION OF INFLAMMATION

Apoptosis is now thought to be key to the resolution of the inflammatory response. Pharmacological manipulation of the rate of apoptosis during chronic inflammatory disorders such as atherosclerosis, may aid the resolution of inflammation and delay disease progression. NO is a promising candidate for such manipulation, because its ability to induce apoptosis and aid inflammatory resolution has already been demonstrated in several animal models. In a mouse model of kidney inflammation, activated macrophages have been shown to induce apoptosis in neighbouring mesangial cells prior to ingestion by phagocytes [75]. The ability of the activated macrophages to induce apoptosis is greatly reduced in the presence of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), indicating that macrophage-directed apoptosis of mesangial cell apoptosis occurs *via* NO-dependent mechanism [76]. Similarly, several studies have demonstrated that activated macrophages infiltrating murine tumours induce apoptosis *via* a NO-dependent pathway in both activated anti-tumour T cells and in the tumour cells themselves [77, 78]. Thus, it appears that macrophages have the capacity to induce apoptosis of nearby cells by the liberation of NO, or a related species, to enhance the clearance of apoptotic cells.

Inducing apoptosis in inflammatory cells within an atherosclerotic lesion is an attractive prospect as it may represent a mechanism to stabilise the plaque or to resolve the vascular inflammation and lead to regression of the plaque, thereby halting disease progression. Reducing the number of activated macrophages present in the plaque would have multiple consequences. Firstly, the physical presence of the plaque in the vessel wall would be decreased, reducing stenosis. Secondly, because activated macrophages induce apoptosis in neighbouring endothelial cells and VSMCs, reducing the number of activated macrophages may help to preserve endothelial function and maintain the integrity of the plaque cap.

NO as a mechanism to regress atherosclerosis is an appealing possibility because in addition to the pro-apoptotic properties described above, NO also has a number of other powerful anti-atherogenic characteristics including inhibition of platelet and inflammatory cell activation [48, 79]. Animal studies are emerging to support the hypothesis that manipulation of apoptosis could be used to reverse atherosclerosis. For example, L-arginine (the substrate for NOS) or the NO donor SNP, administered to hypercholesterolaemic rabbits increases the number of apoptotic macrophages in intimal lesions by three fold [64]. This increase in apoptosis was accompanied by a significant reduction in lesion surface area, suggesting that manipulation of the NO synthase pathway, or delivery of exogenous NO, may be a way to boost NO availabilty in order to stabilise, or even regress, the plaque *via* an apoptotic mechanism. However, the treatments used in this study are by no means selective for macrophages, and as already discussed, NO will also induce apoptosis in endothelial cells and VSMCs. This could have several serious detrimental consequences for the plaque; firstly, additional loss of endothelial function would occur, leading to further exacerbation of the disease process. Secondly, because VSMCs are essential for maintaining the integrity of the plaque cap, loss of this population of cells in vulnerable areas of the lesion could de-stabilise the plaque resulting in plaque rupture which could result in myocardial infarction or stroke. The potential benefit of regressing the plaque in this way must also be offset against the cost of reducing the size of the macrophage population available for scavenging existing apoptotic macrophages, endothelial cells and VSMC. If left *in situ,* these apoptotic cells will undergo secondary necrosis, thereby increasing the thrombogenicity of the plaque as a whole. In the study by Wang *et al.* [64] discussed above, apoptosis is quantified by the number of apoptotic nuclei per area of plaque, rather than as a percentage of the total macrophage population. Therefore, it is not possible to draw any conclusions as to whether the phagocytosis capacity of the plaque has been effected in this case.

Although human macrophages appear unable to generate the supraphysiological concentrations that murine cells produce, human macrophages will respond to exogenous NO delivered by synthetic NO donor compounds, suggesting that NO could potentially be used to manipulate rates of apoptosis in human atherosclerosis. However, it is essential to target any therapeutic intervention to specific cell types within the plaque, and in particular, to appropriate cell types within plaques vulnerable to rupture. Indiscriminate pro-apoptotic events may have serious adverse consequences for the plaque dynamic.

At present, existing NO donor drugs are not selective for particular cell types. Common clinically used organic nitrates, such as nitroglycerin (NTG), tend to have an unfavourable selectivity profile when considered in the context of atherosclerosis, they are more selective for veins (which are not subject to plaque formation) than arteries, and more selective for arteries than platelets (which play a major role in thrombus formation following plaque ruture). In order to use NO donor drugs as effectors of apoptosis in atherosclerosis, compounds which are able to selectively act on, for example, macrophages but not endothelial cells or VSMC, will have to be developed in the future. This however, may be difficult given that macrophages are relatively resistant to oxidative stress induced apoptosis.

In addition to selectivity, a major consideration when contemplating the use of NO donor drugs for the treatment of atherosclerosis is that of delivery. Global, non-selective release of NO throughout the circulation may have various anti-atherosclerotic actions, but dosing will be limited by concurrent vasodilatation resulting in systemic hypotension. The challenge therefore, is to generate high local concentra-

tions in the vicinity of a plaque. One possible means of achieving this is to employ NO donor drugs which are selective for areas of endothelial damage. S-nitrosothiols are generally accepted to be platelet-selective NO donor drugs [80] and have been shown *in vitro* to have vasodilator actions which are selective for areas of experimentally denuded endothelium [81]. Furthermore, in a rabbit balloon angioplasty model of vascular injury, the S-nitrosothiol, S-nitroso-*N*-valerylpenicillamine (SNVP), in contrast to the traditional organic nitrate NTG, reduced the adhesion of radio-labelled platelets in areas of endothelial damage without significantly affecting systemic blood pressure [82]. In this study, the NO donors were delivered directly into the carotid artery *via* a cannula; obviously this is not practical in humans as a long - term therapy, but a possible alternative is to deliver NO *via* drug-eluting stents. There has been some success in humans with the use of sirolimus (rapamycin)-eluting stents in preventing neointimal proliferation (which can lead to restenosis) following angioplasty, although long-term effectiveness (>1 year) remains to be established [83]. A further potential method of delivering high local concentrations of NO directly to the interior of the plaque may be to exploit the lipid nature of the plaque core by developing novel lipophilic NO donor drugs designed to induce apoptosis in the lipid-laden macrophages within the core.

CONCLUSION

Atherosclerosis is now considered a chronic vascular inflammatory disorder involving complicated interactions between many cell types. Apoptosis plays a key role in determining both turnover of cells within the plaque, and plaque vulnerability to rupture. Because apoptotic cells are removed from the site of inflammation without triggering a subsequent pro-inflammatory response, inflammatory cell apoptosis is a promising target for therapeutic intervention in the treatment of this disease. The ability of NO to induce apoptosis, combined with the additional anti-atherogenic properties of NO, points to this molecule being a powerful tool in the treatment of atherosclerosis. The challenge will be to deliver NO in the appropriate chemical form and to balance any benefit in reducing inflammatory cell number within the plaque against the potential deleterious effects of destabilising the plaque cap and promoting plaque rupture.

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